UNIVERSITY OF TASMANIA

Microbial communities associated with bacillary necrosis of Australian blue mussel (*Mytilus galloprovincialis* Lamarck): a study using model larval cultures

by

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tia TASMANIAN INSTITUTE OF AGRICULTURE

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This thesis is dedicated to Felix Kwan who seeded in me the love for science, Agnes Chin and Lucy Chin who mentored me. All inspired me to pursue the scientist’s dream.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Tzu Nin Kwan

(15 November 2017)

Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety
Committees of the University.

Tzu Nin Kwan

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Statement of co-authorship

This thesis includes work, which has been published, submitted or to be submitted for publication in a peer-review journal. The following people and institutions contributed to the publication of work undertaken as part of this thesis:

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Chapter 3. Bacterial community dynamics associated with bacillary necrosis in Australian blue mussel (*Mytilus galloprovincialis* Lamarck) larval rearing induced by overfeeding

Chapter 4. Bacterial community composition and proteolytic activity associated with bacillary necrosis in blue mussel (*Mytilus galloprovincialis* Lamarck) larval culture

Chapter 5. *Pseudoalteromonas* sp. : an opportunistic pathogen of Australian blue mussel (*Mytilus galloprovincialis* Lamarck) larvae causing velar deformation and detachment

Tzu Nin Kwan designed and carried out the experiments, collected and analysed the samples, carried out the data analysis, and wrote the manuscript. Christopher J.S. Bolch and John P. Bowman provided guidance on experimental design, data analysis and interpretation, and edited drafts of the manuscript.

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Abstract

Bacillary necrosis is a sporadic and rapid bacterial disease, capable of causing total collapse of larval cultures (known as larval crash) in a period of 24-48 h and is today, the most prevalent hatchery disease worldwide, affecting more than 20 bivalve species. The prevailing view is that *Vibrio* are primary pathogens even though this is based on limited understanding of direct causality and natural infection. As a result, this first part of the study investigated population dynamics and the role of culturable *Vibrio* in spontaneous outbreaks of bacillary necrosis in larval culture of Australian blue mussel (*Mytilus galloprovincialis*) reared under laboratory conditions.

Given the importance of organic matter in regulation of *Vibrio* concentration in hatchery environments, the study used overfeeding of microalgae to induce bacillary necrosis. *Vibrio* proliferated at the beginning of overfed cultures by >20 % relative abundance. The patterns of change in *Vibrio* relative abundance in seawater and larvae coincided with onset of mortality (daily mortality >30 %) and progressed to a cumulative mortality > 70 % by 8 dpf. In contrast, standard fed cultures showed cumulative mortality no more than 15 %. *Vibrio* was not routinely detected in larvae samples except on the beginning of mortality and ranged from 2.8 to 4.7 x 10^2 CFU $larva^{-1}$. The majority of culturable *Vibrio* were members of the *Vibrio splendidus* clade, a diverse bacterial group that is often implicated as causative agents. Interestingly, despite its increased relative abundance with mortality, challenge bioassays by immersing larvae in seawater with a series of bacterial cell concentration suggest these *Vibrio* isolated from the diseased cultures were unlikely pathogens. Therefore, this study in contrary to many published studies shows that *Vibrio splendidus* proliferation during mortality events can also result from growth of *Vibrio* communities normally associated with larval cultures and may not have a direct role in disease.

The second part the thesis then characterised the bacterial communities from the same overfeeding experiment using molecular techniques. A total of 72 seawater and larvae samples (of which 6 associated with mass mortality) were analysed using Automated Ribosomal Intergenic Spacer Analysis (ARISA) of 16-23S rDNA intergenic spacer 1 (ITS1). Canonical analysis of principal coordinates (CAP)

examining samples (n=72) categorised into normal, a day before (n=2), during (n=2) and a day after mortality (n=2) demonstrated systematic shifts in seawater and larval bacterial communities corresponding to the infection course of bacillary necrosis as mortality developed and subsided. Abnormal bacterial communities initiated in seawater up to 3 d prior to onset of larval mortality, and the timing of mortality coincided with the uptake of seawater communities by the larvae. This study for the first time demonstrated bacillary necrosis is potentially initiated in seawater even though the onset of bacillary necrosis remains unpredictable in both timing and scale of larval mortality. This suggests seawater is a potential important source of variation.

In order to examine the link of seawater associated bacterial communities with variability in bacillary necrosis, a study was instigated that examined nineteen replicate small-scale larval cultures using 0.22 µm filtered seawater and a commercial algal food to minimize variation. Despite strict replication of larval cultures, mortality events occurred unpredictably in four of the nineteen cultures. Bacterial community analysis using Illumina MiSeq derived 16S rRNA gene taxa-abundance data showed mortality events were consistently linked with a similar group of bacteria. Linear discriminant analysis effect size (LEfSe) focusing on larval community data show sequence reads affiliated with *Marinomonas* sp. and *Gracilibacteria* were statistically more abundant in high mortality larval whilst *Pseudoalteromonas distincta*, *Phaeobacter gallaenciensis* and *Vibrio splendidus* were more abundant in low mortality cultures. This study also examined proteolytic activity in the larval culture seawater and for the first time showed a significant association between total proteolytic activity of rearing seawater and mortality. So far, this thesis observed that multiple bacteria can be associated with bacillary necrosis, and examinations of the interactions of some of these bacteria with mussel larvae may help progress our understanding of bacillary necrosis.

The final part of this thesis investigated how bacteria isolated during outbreaks of bacillary necrosis interact with larvae in challenge assay conditions. In this study, ten bacterial colonies from the highest diluted plates (10^{-7}) of Marine Agar (MA) were randomly isolated during a mass mortality event in laboratory larval cultures, and screened for virulence using mussel larval bioassays. A series of larval challenge

bioassays shows these isolates possessed moderate virulence (LC_{50} 3.2±2.1 × 10^4 cells ml^{-1} at 2 day post challenge (dpc) of 6 dpf larvae in 0.22 µm filtered seawater). Interestingly, loss of virulence was observed for repeated sub-cultures. Comparison of 16S rRNA gene sequences showed that all 10 isolates were identical and represented an undescribed species of the genus *Pseudoalteromonas*, indicative that this bacterial strain occurred in higher concentration in the larval culture suffering mass mortality. Histopathological examinations identified early, mid, severe, completion and post mortem stage of disease development across 3 d characterised by cleavage and detachment of velum without direct invasion of the velar tissue by bacterial cells indicative of destruction potentially mediated by toxins. However, marine-milk agar and azocasein assays detected only limited proteolytic activity of the *Pseudoalteromonas* isolates. Whilst more research is necessary, this study demonstrates for the first time a *Pseudoalteromonas* strain has been shown to negatively implicate mussel larval cultures.

This thesis concludes, based on the model larval cultures that members of the *Vibrio splendidus* group have close links with bacillary necrosis however not necessarily as direct cause of larval mortality. Molecular techniques further show representation of *Vibrio* fades in the community wide changes suggestive of more complex interactions of bacteria than anticipated in shaping causality. The unpredictability and lack of association of mortality episodes with specific bacterial groups adds weight to the idea that bacillary necrosis is a condition associated with imbalance compositional changes in seawater or larval bacterial communities that can promote opportunistic interactions and influence larval health outcomes. The relevance of opportunistic pathogenesis/dysbiosis is emphasized here which adds another dimension of complexity to the current view of bacillary necrosis.

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List of Abbreviations

16S rRNA 16S ribosomal ribonucleic acid

ANOVA analysis of variance

ARISA Automated ribosomal intergenic spacer analysis

bacillary necrosis baciallry necrosis

BLAST Basic Local Alignment Search Tool

bp base pair

CAP canonical analysis of principal coordinates

CFU colony-forming unit

dpc day post challenge

dpf day post fertilisation

df degree of freedom

DGGE Denaturing Gradient Gel Electrophoresis

DNA deoxyribonucleic acid

DOM dissolved organic matter

ECPs extracellular products

LC_{50} lethal concentration causing 50 % mortality

LEfSe linear discriminant analysis effect size

m number of principal coordinates

MA Marine Agar

MDS multidimensional scaling

NCBI National Centre for Biotechnology Information

NGS next generation sequencing

NMDS Nonmetric multidimensional scaling

OTU Operational taxonomic unit

PCO Principal Component Analysis

PCR polymerase chain reaction

PERMANOVA permutation multivariate analysis of variance

RNA ribonucleic acid

SAS Statistical Analysis System

spp. species

TCBS thiosulfate-citrate-bile salts-sucrose

TRFLP terminal restriction fragment length polymorphism

UV ultra violet

vsm metalloprotease gene

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Chapter 1 Introduction

1.1 Global and Australian seafood production

Seafood is defined as food sourced from aquatic environments whether by means of capture or aquaculture, and this includes fish, crustaceans, mollusc, other aquatic animals and plants. Total global seafood production recorded 160 million tonnes in 2014 of which aquaculture contributed approximately 45 % (FAO, 2016). Aquaculture for human consumption is the fastest growing primary industry in the world. Global aquaculture production has seen an exponential growth reaching well over 100-fold increase in production volume from 60,000 tonnes in 1950 to over 64 million tonnes 2011 (FAO, 2016).

Australian aquaculture has followed a similar trend and is an increasingly important economic generator. In 2016 aquaculture contributed 50 % (80,004 of 157,467 tonnes) of total national seafood production and was valued at $1 billion (FAO, 2016). While 85 % of Australian aquaculture production comes from five high value species; southern Bluefin tuna (*Thunnus maccoyii*), pearl oysters (*Pinctada maxima*), Atlantic salmon (*Salmo salar*), edible oysters (*Crassostrea gigas* and *Saccostrea glomerata*) and prawns (*Penaeus monodon* and *Fenneropenaeus merguiensis*), there are tremendous developmental opportunities for other species as the world's appetite for Australian seafood is increasing. The need for diversification of aquaculture species is recognised with collaborative research and development projects already underway.

1.2 Australian mussel aquaculture

Molluscan aquaculture production in 2012/13 has increased by 25 % since 2002/03 and constitutes approximately 10 % of national aquaculture by value (Skirtun et al., 2013). The species cultured are mainly edible oysters while mussel still represents an emerging shellfish product. The accepted commercial common name of the mussel cultured is the Australian blue mussel (*Mytilus galloprovincialis*).

Australian blue mussels are farmed in all states of Australia except Northern Territory and Queensland. National production has been stable at around 3,500 tonnes since 2010, and the sector is valued at around $10 million. Tasmanian production accounts for 30 % of national production (Skirtun et al., 2013). Tasmanian mussel production is concentrated in the south around Cygnet, Port Arthur, Dover and Spring Bay.

Production of mussels are still considered minimal because Australia in 2012/13 imports a further 2,400 tonnes of frozen mussels whilst neighbouring New Zealand exported $180 million worth of farmed Greenshell mussels (*Perna canaliculus*) in 2013 (Skirtun et al., 2013). This indicates potential for the Australian mussel aquacultures to increase production for both local and domestic markets.

Unlike other farmed bivalves, mussels require settlement substrate for attachment. Farming of mussel uses longline system (**Figure 1-1**a), and mostly still rely on wild collected mussel seeds or spat. However, the sourcing of mussel spat from the wild imposes limits to the capacity of the mussel aquaculture industry to increase

production, control quality of products and timing of supply to markets. Reliance on wild spat ultimately leaves the industry vulnerable to spat recruitment failure, restricts production to seasonal availability and limits capacity to develop selective breeding programs.

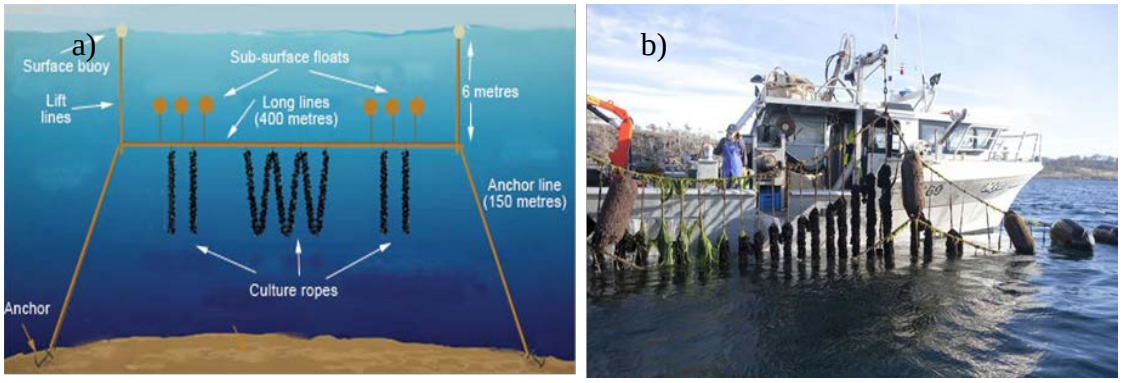
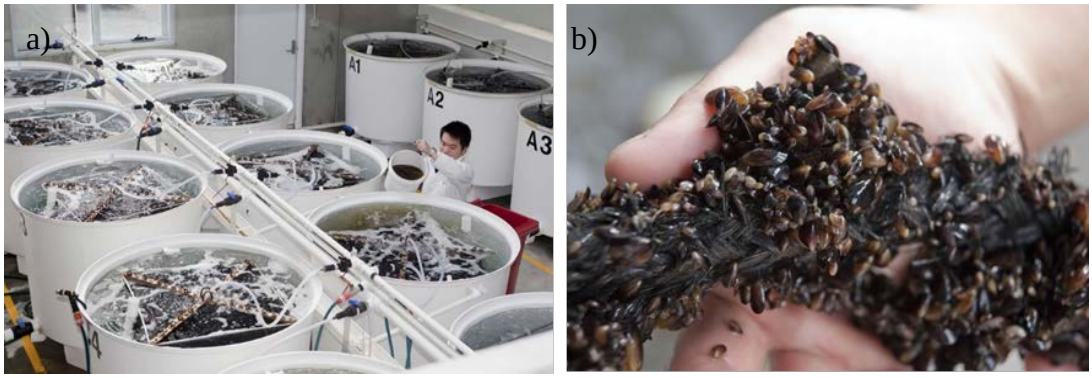


Figure 1-1. a) The longline system is made up of thick horizontal long ropes called "backbones" usually up to 400 m long and secured at both ends of anchors. The "backbones" are suspended in water column by a series of sub-surface floats. Culture ropes or "droppers" which is the attachment substrate for mussels is connected to the "backbones" in single drop down or loop fashions. This ensures mussels are cultured well above the bottom surface and receive good tidal exchange. Most part of the long line system is submerged and is marked by surface buoys. b) the mussels are harvested by mechanically "striping" the droppers to detach mussels from the ropes. This system allows highly mechanised farming and yields up to 10-20 t per hectare per year (Images reproduced with permission from Phil Lamb, Hatchery Manager of SBS, Triabunna).

Mussel industries in Tasmania and Victoria have taken steps to minimise reliance on wild spat collection through development of hatchery produced mussel spat. Spring Bay Seafoods (SBS), a commercial mussel operators in Triabunna have developed larval and spat rearing techniques to produce their own spat to supply all stock for adult production (**Figure 1-2**).

While the physical technology, processes and husbandry is similar to that used for larval oyster production, broodstock conditioning and settlement (metamorphosis) utilize different technology. As with oysters, the larval rearing phase is subject to unpredictable and unexplained mortality. As a result specific research is required to improve quality and survival of mussel for commercial production.



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1.3 Mortality of hatchery reared bivalve larvae and spat

Bivalve are constantly exposed to high concentration of microbes in seawater and their microbial interaction is enhanced by their filter feeding that concentrates bacteria from the surrounding water (Beleneva et al., 2003; Pruzzo et al., 2005).

The microbial environment in which bivalves live can have a substantial influence on bivalve health. Changes in the microbial community can cause stunted growth, developmental abnormalities and increased mortality. This risk is exacerbated in bivalve hatcheries as animals are often held in a confined volume with high organic

waste concentration derived from culture animals and feed inputs (Helm et al., 2004). Such combination can result in rapidly changing microbial population in the rearing environment therefore can destabilise larvae-microbial interaction (Walne, 1958; Schulze et al., 2006).

Hatchery production worldwide has been characterised by diseases caused a range of microbes. So far mortality events have been associated with both bacteria (Tubiash et al., 1970; Lodeiros et al., 1987; Lodeiros et al., 1992; Elston, 1993; Sugumar et al., 1998b; Elston, 1999; Elston et al., 2008b; Prado et al., 2015) and viruses (Elston and Wilkinson, 1985; Hine et al., 1998; Hwang et al., 2013) although there is more substantial published evidence implicating bacteria as the main etiologic agent. This is also reflected in the diverse range of bacterial strains that affect all hatchery reared bivalve species. Conversely, viral diseases has to date only been implicated in hatchery mortality of ostreid bivalves (Elston and Wilkinson, 1985; Hine et al., 1998; Renault et al., 2000; Hwang et al., 2013).

The associations of bacteria with poor survival of hatchery reared mollusc larvae have been observed as early as the 1950s in the United States when rearing techniques were trialled in the laboratory (Walne, 1956; Guillard, 1959). Today, bacterial infections are still regarded as the most serious disease of hatchery-reared larvae (Nicolas et al., 1996; Paillard et al., 2004; Elston et al., 2008b) causing rapid and complete mortality, colloquially known as a larval crash. Whilst it is difficult to quantify the impact of bacterial diseases in shellfish hatcheries, frequent recurrence can severely impact hatchery productions with repercussions often felt throughout the supply chain.

1.4 Bacillary necrosis and bivalve larval rearing

Investigations of larval mortality in some of the earliest intensive larval cultures of clams (*Mercenaria mercenaria*) and oysters (*Crassostrea virginica*) observed bacterial infection leading to necrotised larval tissues. In all cases Gram negative bacilli were associated with mortality (Takeuchi et al., 1956; Guillard, 1959; Tubiash et al., 1965; Tubiash et al., 1970). Intuitively, Tubiash et al. (1965) categorised these abnormal larval conditions under the broad term of bacillary necrosis and later attributed them as a bacterial disease caused primarily by species of the genus *Vibrio*.

Despite refinement of rearing techniques, bacillary necrosis still affects all commercial bivalve hatcheries. Bacillary necrosis is the most prevalent disease in larval cultures (Table 1-1) and capable of causing total collapse of larval cultures within 24-48 h (Jeffries, 1982; Garland et al., 1983). With increasing hatchery production, bacillary necrosis is also increasingly reported worldwide from more than 20 bivalve species (Table 1-1) across North America, Europe, Asia and Australia making bacillary necrosis the most serious bacterial disease in larval rearing (Elston and Leibovitz, 1980). Affected species includes scallop (family *Pectinidae*; *Argopecten irradians, Argopecten purpuratus , Argopecten ventricosus, Euvola ziczac*, *Nodipecten subnudosus*, *Pecten maximus*), clam (family *Veneridae*; *Mercenaria mercenaria*, *Ruditapes decussatus*, *Venerupis philippinarum*), cockle (family *Cardiidae*; *Fulvia mutica*, *Tricadna gigas*), penshell (family *Pinidae*; *Atrina maura*), oyster (family *Ostreidae*; *Crassostrea virginia*, *Crassostrea gigas, Crassostrea sikamea, Ostrea edulis*), mussel (family *Mytilidae, Mytilus galloprovincialis, Perna canaliculus*), and geoduck (family *Hiatellidae, Panope*

abrupta).

Research so far demonstrated bacillary necrosis is associated with a wide range of bacterial species belonging to the genus *Vibrio*. Hence, the disease is often referred to as “vibriosis”. The pathogenic bacteria includes strains of *V. alginolyticus* (Anguiano-Beltran et al., 2004; Gomez-Leon et al., 2005), *V. coralliilyticus* (Kesarcodi-Watson et al., 2009a; Richards et al., 2015), *V. pectinicida* (Lambert et al., 1998), *Vibrio splendidus* (Sugumar et al., 1998b; Gomez-Leon et al., 2005; Saulnier et al., 2010), *V. tapetis* (Paillard et al., 2008) and *V. tubiashi* (Lodeiros et al., 1987; Elston et al., 2008b; Richards et al., 2015; Prado et al., 2015). The high numbers of reports associated *Vibrio* bacteria with bivalve disease could be partly attributed by easy culturability on nutrient media and their ubiquitous presence in coastal environment, and often associated with marine organisms (Brown and Losee, 1978; Sugumar et al., 1998b). These studies followed the Koch’s postulates to establish causative agents but under the assumption that the aetiologic agent was unique and present in moribund or dead animals at high concentration.

Table 1-1. Updated summary of virulent bacteria associated with bacillary necrosis in hatchery reared bivalve larvae (adapted from Paillard et al., 2004 and includes an additional of 10 new references up to 2015).

Locations	**Pathogen**	**Host**	**References**
United States	*Aeromonas* sp.	*Crassostrea virginia, Mercenaria mercenaria*	(Tubiash et al., 1965)
	Vibrio anguillarum, V. alginolyticus	*C. virginia, M. mercenaria, Ostrea edulis,*	(Tubiash et al., 1970)
	Vibrio anguillarum	*Crassostrea gigas*	(Disalvo et al., 1978)
	V. alginolyticus	*Atrina maura, Nodipecten subnudosus, C. gigas*	(Luna-Gonzalez et al., 2002)
	V. tubiashii	*Agropecten irradians, C. virginia*	(Tubiash et al., 1965; Richards et al., 2015)
		M. mercenaria	(Hada et al., 1984)
		C. gigas, Crassostrea sikamea, Panope abrupta	(Elston et al., 2008b; Richards et al., 2015)
		C. virginia	(Brown, 1973; Leibovitz, 1978)
	Vibrio spp.	*M. mercenaria, C. virginia*	(Brown and Tettelbach, 1988)
	Pseudomonas sp.	*C. virginia*	(Brown, 1973)
		M. mercenaria	(Guillard, 1959)
		C. virginia	(Brown, 1981a)
Spain	*V. anguillarum*	*O. edulis*	(Lodeiros et al., 1987)
	V. alginolyticus	*Ruditapes decussatus*	(Gomez-Leon et al., 2005)
	V. neptunius	*O. edulis*	(Prado et al., 2005)
	V. tubiashii	*O. edulis*	(Lodeiros et al., 1987; Prado et al., 2015)
	V. ostreicida	*O. edulis*	(Prado et al., 2014b)
		Venerupis philippinarum	(Prado et al., 2015)
	Pseudomonas sp.	*O. edulis*	(Lodeiros et al., 1987)
		Euvola ziczac	(Lodeiros et al., 1992)
France	*V. pectenicida*	*Pecten maximus*	(Lambert et al., 1998)
	V. splendidus	*P. maximus*	(Nicolas et al., 1996)
		R. decussates	(Gomez-Leon et al., 2005)
Chile	*Aeromonas hydrophila*	*Argopecten purpuratus*	(Riquelme et al., 1996)
	V. aginolyticus	*A. purpuratus*	(Riquelme et al., 1996)
	V. anguillarum	*A. purpuratus*	(Riquelme et al., 1995)
Britain	*Vibrio* spp.	*C. gigas, O. edulis*	(Jeffries, 1982)
Norway	*V. splendidus*	*P. maximus*	(Torkildsen et al., 2005)
Japan	*V. splendidus*	*Fulvia mutica*	(Fujiwara et al., 1993)
		C. gigas	(Sugumar et al., 1998a)
Australia	*V. campbelli, V. harveyi, V. orientalis, V. splendidus biotype I, V. damselae, V. alginolyticus, Vibrip* spp.	*Tricadna gigas*	(Sutton and Garrick, 1993)
	Vibrio spp.	*C. gigas*	(Garland et al., 1983)
	Alteromonas spp.	*C. gigas*	(Garland et al., 1983)
New Zealand	*V. splendidus*	*Perna canaliculus*	(Kesarcodi-Watson et al., 2009a; Kesarcodi-Watson et al., 2009b)
	V. coralliilyticus/neptunius	*P. canaliculus*	(Kesarcodi-Watson et al., 2009a; Kesarcodi-Watson et al., 2009b)
Mexico	*V. alginolyticus*	*Mytilus galloprovincialis*	(Anguiano-Beltran et al., 2004)
		Argopecten ventricosus	(Sainz et al., 1998)

1.5 The terminology: bacillary necrosis

Despite well over 50 years since Tubiash et al. (1965) coined the term bacillary necrosis, the definition has not been redefined to reflect what is a disease with diverse characteristics. So far, most cases are currently amalgamated together under the board term of bacillary necrosis despite variations of causative agents, symptoms and pathology. It could be for these reasons the term has not been widely used instead alternative descriptions such as larval crash (Richards et al., 2015), larval necrosis, vibriosis (Leibovitz, 1979; Paillard et al., 2001; Paillard et al., 2004) and acute pallial infection (Elston, 1999) have been used. These descriptions are valid in its specific contexts, but a more thorough understanding of the disease is lacking.

1.6 Characteristic of bacillary necrosis

The majority of investigations of bacillary necrosis occur in response to increased or complete larval mortality. As a result the literature describes many individual pathogens but there remains little understanding of the disease as a whole. However, despite the diversity of affected hosts and pathogens, there are several common characteristics and features of the disease.

1.6.1 Larval swarming

Hatchery rearing staff often report observations of a distinctively abnormal larval behaviour known as larval aggregation, referred to as “larval spotting” or “larval swarming” prior to onset of bacillary necrosis (see Disalvo et al., 1978; Sugumar et al., 1998b; Prado et al., 2005; Chapman, 2012; Bryce Daly pers. Comm.). In fact, Chapman (2012) reported six out of seven mass mortality events in Pacific oyster

larval production runs in Tasmania were preceded by larval swarming. The larvae are often observed to descend in the water column to near the bottom of the tank despite water circulation, and form dense swarming aggregations. This behavioural change represents the earliest clear indication of subsequent disease.

So far, the cause of swarming behaviour and the link to mortality events have not been directly demonstrated but Sugumar et al. (1998b) reported successful application of streptomycin to help prevent swarming behaviour and subsequent successful completion of larval development. Bioassay challenges of extracellular products from pathogenic *Vibrio splendidus* and *Vibrio anguillarum* to healthy larvae resulted in loss of swimming ability due to arrest of velar ciliary beating causing the larvae to settle to the bottom of tank (Disalvo et al., 1978; Sugumar et al., 1998b). The cessation of ciliary beating has been attributed to low molecular weight (0.5 to 1 kDa) ciliostatic toxins produced by various strains of *Vibrio* (Nottage and Birkbeck, 1987; Nottage et al., 1989).

Whether ciliostatic toxins are involved in swarming behaviour is unclear. Chapman (2012) observed swarming larvae to have a high level of ciliary activity of and to be active swimmers apparently free of disease. Swarming may therefore be an active, synchronised behaviour rather than passive sinking.

1.6.2 *Vibrio* concentration and growth spikes

The majority of bacteriological studies of bacillary necrosis target *Vibrio* species due to the relatively easy recoverability of *Vibrio* on selective nutrient media (such as thiosulfate-citrate-bile salts-sucrose, TCBS). However, culture-independent

molecular techniques show that despite high concentrations, the relative abundance of *Vibrio* is often less than 1 % of total bacteria during a mortality event (Jorquera et al., 2004; Chapman, 2012; Powell et al., 2013). It is possible that the importance of *Vibrio* as a causative agent may be over-estimated and that the rest of the microbial community may interact with pathogens in the aetiology of the disease.

Alternatively, the concentration and specific type of *Vibrio* bacteria may have more direct importance in pathogenesis than their proportion in the total population. Some studies indicate that hatchery larval mortality is associated with *Vibrio* concentrations above log 4 CFU ml^{-1} (Nicolas et al., 1996; Sugumar et al., 1998b; Lacoste et al., 2001). These include *Vibrio tubiashii*, *Vibrio splendidus* and *Vibrio coralliilyticus* (Kesarcodi-Watson et al., 2009a; Richards et al., 2015). In fact, De Decker et al. (2013) shows that a conditioned culture medium prepared from supernatant of an avirulent *V. splendidus* is capable of enhancing virulence in a different species *Vibrio aestuarianus*, presumably via interspecific bacterial cell density linked quorum sensing. Given the example of inter *Vibrio* species interaction in modulation of virulence, *Vibrio* growth spikes could have indirect roles in facilitating virulence in bacillary necrosis.

Culturing techniques have been useful in detecting changes in *Vibrio* populations associated with bacillary necrosis. Sugumar et al. (1998b) detected 35 % and 50 % rise in the culturable *Vibrio* abundance in seawater and larvae of Pacific oyster larval culture respectively that exhibited bacillary necrosis. Similarly, Chapman (2012) detected a 3 log increase of culturable *Vibrio* (reaching 35 % relative abundance) in seawater of larval culture that later developed bacillary necrosis.

Such shifts are generally hard to detect by assessment of *Vibrio* concentration alone because some are attributed to decrease in total viable cells rather than the increase of counts (see Nicolas et al., 1996). Yet, these *Vibrio* population changes are significant but often short lived, leading to a situation described herein as *Vibrio* growth spikes. So far, no study has investigated the nature of the growth spikes in in seawater and larvae, and genetic composition of the spikes and roles in bacillary necrosis if any. Previous studies such as (Hidalgo et al., 2008; Prado et al., 2014a; Kwan and Bolch, 2015) showed representativeness of *V. splendidus* related bacteria in many bivalve hatcheries, therefore suggestive of their association with *Vibrio* peaks.

1.6.3 Dynamic bacterial communities associated with larval cultures

More recent bacteriological studies of bacillary necrosis have extended beyond pathogen identification to appreciate the role and influence of the wider bacterial communities associated with bivalve hatchery culture environments. Mussel hatchery cultures harbour a huge genetic diversity of culturable *Vibrio* strains typically dominated by *V. splendidus*-related genotypes (Kwan and Bolch, 2015). More than 40 unique *atp*A gene sequences belonged to the *V. splendidus* group were detected among 87 isolates examined, indicative of high genotypic diversity which otherwise would not be detected using the 16S rRNA gene. Relative abundance and composition of these genotypes differed in different parts of the hatchery water supply and hatchery rearing stage. Even though limited to culturable populations of *Vibrio*, this work shows genotype relative abundance and dominance is highly

dynamic, and may change rapidly during different stages of rearing. The rich genetic diversity of bacteria in hatchery culture environments may present problems to accurately describe bacterial communities. This is because genomic variation of related genotypes are insufficiently reflected by examination of a small region of gene sequence (particularly the 16S rRNA gene). This would result in the inability to detect specific important genotypes that otherwise would improve correlation with bacillary necrosis.

Seawater bacterial communities associated with hatchery culture of oysters varies on several scales: over days within the same larval rearing period (i.e 3 weeks), production runs within one season (5 month period) and across production runs over a year (Powell et al. 2013). Using terminal restriction fragment polymorphism (tRFLP) and tag encoded amplicon sequencing, the authors showed lack of correlation of microbial communities with larval mass mortality and attributed this to complex and transient changes in water quality and rearing conditions.

The dynamic nature of bacterial community in larval rearing environment could suggest that bacillary necrosis is a common description of necrotic infection caused by a diverse group of bacteria (see Table 1-1).

1.6.4 Pathogenesis of bacillary necrosis

The disease symptoms of bacillary necrosis observable under a dissecting microscope are generally similar in infected larvae of various species (Elston, 1999). The combination and extent of pathology vary according to severity of disease but in most cases, would include a transparent gut indicating reduced feeding activity,

abnormal swimming behaviour presented as short, erratic swimming in tight circular motion with sporadic cease of swimming, high activity level of velar protrusion and/or abnormal morphological changes in the swimming accessories including distortion of velum, reduced or rounding of velum with loss of cilia, and in some cases, disintegration of velum (Tubiash et al., 1965; Sainz et al., 1998; Travers et al., 2015). The advanced stage of bacillary necrosis involves gross larval tissue lessions with subsequent death.

These symptoms can be easily identified hence have been widely reported. However, their generic definition and relatedness suggest a common suite of symptoms and disease progression. However, in depth examination by Elston and Leibovitz (1980) using live animal examination, immunological testing, histology and electron microscopy revealed different infection routes and types of deterioration in larvae indicative of variable pathogenesis. It is surprising that very few studies has considered or used the pathological descriptions, reflecting the lack of awareness of infection dynamics and the current poorly characterised pathogenesis. Elston and Leibovitz (1980) demonstrated 3 patterns of pathogenesis of bacillary necrosis, Type I, II and III, caused by *Vibrio* strains whose advanced stages presented overlapping pathologies. Briefly, Type I pathogenesis, known as acute pallial infection (Elston, 1999) affect all larval stages, describes attachment and proliferation of causative bacteria on and along the shell-mantle tissue margin before invading the visceral cavity. Velum and digestive tracts are generally unharmed in the early stage. The Type I pattern appears the most common and has been reported by many studies (Brown and Losee, 1978; Elston et al., 1981; Sugumar et al., 1998b; Gomez-Leon et

al., 2005).

Type III pathogenesis, affects late veliger (19 to 22 dpf) and initiates by disruption of digestive gland absorptive cells before resulting in necrosis in organs and digestive tracts (e.g. Garland et al., 1983; Nicolas et al., 1996). Also, Kesarcodi-Watson et al. (2009a) reported similar pathology in reared mussel larvae. The rarer observation of abnormal deposition of lipid granules in the digestive system (see Sainz et al., 1998) is related to this pathogenesis type. Both Type I and III infected larvae at early stage of infection are often found on bottom substrate and show less active organ activity.

In contrast, Type II infections affect planktonic larvae initially who display erratic and tight circular swimming motions, and velar deformities such as an inability to retract the velum and/or deciliation or clumping of velar tissues, but otherwise exhibit fully active organ activity. Type II pathogenesis occurs in the absence of bacterial invasion of tissues. Elston (1999) attributed this to remote virulence caused by toxic metabolites from bacteria hence is alternatively known as toxin-induced velar deformation. The most recent outbreak of Type II bacillary necrosis was reported by Elston et al. (2008b) and the aetiological agent was *V. tubiashii*.

1.6.5 Virulence mechanisms

Since bacillary necrosis is often associated with bacteria belonging to the genus *Vibrio*, a considerable proportion of bacillary necrosis-related research has focussed on pathogenicity of *Vibrio* species. Pathogenicity studies using bioassays reveal that *Vibrio* virulence can also be attributed to cell free supernatant produced from pure cultures because it produced mortality levels comparable with those that used washed

bacterial cells (Jeffries, 1982; Sugumar et al., 1998a; b; Gomez-Leon et al., 2005; Saulnier et al., 2010). This suggests that extracellular products (ECPs) presumably containing toxins are also an important factor in facilitating lethality without direct interaction of bacterial cells and larval tissues, to cause necrosis and/or facilitate bacterial invasion.

Invasion of host tissue by bacteria involves a range of mechanisms such as surface attachment, ability to overcome hosts' immune system and production of invasins including proteases. Metalloprotease production is associated with *Vibrio*-related virulence to marine animals. For example, metalloprotease secretion is necessary to cause tissue lesions in tiger prawns by *V. harveyi* (Teo et al., 2003), invasion of the fish host by *V. anguillarum* (Norqvist et al., 1990), degradation of gill tissues of blue mussel (Nottage and Birkbeck, 1987), and photo-inactivation and coral tissue lesions by *V. shiloi* and *V. coralliitycus* (Sussman et al., 2009). The ECPs from isolates of *V. fluvialis*, *V. mimicus*, *V. vulnificus*, *V. cholerae*, and *V. tubiashi* ,and *V. splendidus* have also been shown to be responsible for >70 % of the mortality of oyster larvae (Hasegawa et al., 2009). The toxicity of the ECPs could also be substantially reduced by addition of the chelating compound ethylenediaminetetraacetic acid (EDTA). All 6 strains carried homologs of metalloprotease gene complexes and mutants devoid of the metalloprotease gene by counter-selectable suicide vector showed reduced toxicity of the ECPs to oyster larvae, indicating that metalloprotease are a common virulence factors in many shellfish pathogenic *Vibrio* (Hasegawa et al., 2008).

Studies using bioassays, sequence comparisons and genetic manipulations demonstrated metalloprotease as a major virulence factor in the ECPs of several

important pathogens capable of bacillary necrosis including *V. tubiashii* ATCC190105, RE22 and RE98 (Hasegawa et al., 2008), *V. aestuarianus* 01/32 (Labreuche et al., 2010; Saulnier et al., 2010), and similarly *V. splendidus* (Le Roux et al., 2007; Binesse et al., 2008; Hasegawa et al., 2009; Saulnier et al., 2010).

Binesse et al. (2008) demonstrates the active constituent of the ECPs by exposing purified metalloprotease from its ECPs to mollusc cell lines and juvenile oysters. The authors observed severe cytotoxic effects and lethality of the purified metalloprotease indicating its direct role in contributing to ECP toxicity as crude products resulted in marginal increase in toxicity. Even though these studies did not examine other potential toxins that may be present in the ECPs, inhibition of haemolysin produced by *V. tubiashii* did not substantially supress lethality to oyster larvae, further supporting the primary contribution of metalloprotease to the toxicity of ECPs (Hasegawa et al., 2008).

In view of the facts that *V. splendidus* genotypes are typically the dominant *Vibrionaceae* and most often associated with larval mortality in bivalve larval cultures (Sugumar et al., 1998b; Kwan and Bolch, 2015; Rojas et al., 2015), more research focuses on metalloprotease production in *V. splendidus*. Crucial to the subsequent development of understanding in virulence factors in *V. splendidus* is the completion of genome sequences (Le Roux et al., 2009) where a metalloprotease gene (*vsm*) was later confirmed to encode for zinc metalloprotease. The availability of genome data has made possible comparison with known virulence genes to discover and investigate for virulence factors. Amino acids sequence alignment of zinc metalloprotease of *V. splendidus* showed 95 % and 88 % homology respectively

to those produced by pathogenic *V. anguillarum* (Le Roux et al., 2007) and *V. tubiashii* (Hasegawa et al., 2008). The latter authors further found that a closely related but avirulent strain of *V. tasmaniensis* LMG20012^T lacked the metalloprotease gene.

Genetic manipulation of the *vsm* gene offered strong evidence of the crucial role of this gene in virulence of *V. splendidus*. Regulation of expression of the *vsm* gene in *V. splendidus* LPG32 corresponds strongly to its toxicity and deletion of the *vsm* gene results in substantial decrease in toxicity of the pathogen. (Le Roux et al., 2007; Binesse et al., 2008). Using different approaches, they further showed that insertion of the *vsm* gene by allelic exchange into an avirulent strain *V. tasmaniensis* LMG20012T, dramatically increased lethal effects of its ECPs to oyster larvae (Hasegawa et al., 2008). By performing comparative genome analysis, Le Roux et al. (2009) found that three strains of *V. splendidus* possess different combinations of putative virulence genes such as the InA-like metalloprotease and *vas* operon but the *vsm* gene is shared by all strains indicating commonness of the *vsm* gene in *V. splendidus*. The involvement of metalloproteases in virulence is also supported by a large scale epidemiological study (Saulnier et al., 2010). Using oyster larval assays and azocaseinase activity a high level of correlation was observed between protease activity and strain virulence in *V. splendidus* strains isolated from various hatcheries in France. These studies all indicate that metalloproteases are a major virulence factor in *Vibrio* capable of causing bacillary necrosis in larval shellfish.

1.7 Bacillary necrosis in Tasmanian mussel hatcheries

Mussel larval stock mortalities are a particular problem in a commercial Tasmanian blue mussel hatchery. Major losses tend to occur during the first week of planktonic stage of larvae (first week post fertilisation) and near metamorphosis (2-3 weeks post fertilisation). Preliminary studies of larval mortality indicated bacillary necrosis associated with *Vibrio*. Studies of culturable *Vibrio* in the hatchery indicate that the culturable *Vibrio* community is dominated by *V. splendidus*, and is highly dynamic, varying significantly at various points in the seawater supply and larval culture systems in the hatchery (Kwan and Bolch, 2015). These studies suggested that hatcheries are a highly dynamic microbial environments where husbandry practices such as water exchange, changes in algal food quality and quantity modify the quality and quantity of organic carbon throughout larval production. These changes may lead to rapid shifts in microbial community and/or *Vibrio* genotype dominance, leading to bacillary necrosis. However, it remains unclear how larval husbandry and seawater quality affects the microbial community associated with larval cultures, or whether these changes lead to increasing incidence of bacillary necrosis.

While there is substantial published work detailing causative agents of bacillary necrosis, particularly *Vibrio* species there is little understanding of the disease within the broader microbial community context. The need to widen the view from single pathogens to microbial communities is necessary because behaviour of pathogens are complex as a result of interactions among the bacterial community. Outbreaks of diseases in commercial settings are unpredictable to the extent many commercial operators describe them as random events (Elston et al., 2008b; Chapman, 2012).

The poor correlation of presence/concentration larval pathogens and disease indicates that most cases of bacillary necrosis are not simple single pathogen-host interactions. In fact, numerous studies reported co-existence of pathogenic bacteria and larvae host without disease incidence (Brown, 1981b; Schulze et al., 2006; Elston et al., 2008b). Therefore in a commercial context where pathogens are not deliberately introduced to larvae, bacterial diseases are perhaps ecological events brought about by changes in microbial communities that present lethal interactions to bivalve larvae. This may include, but is not limited to, changes that allows proliferation of pathogens, or suppression of beneficial bacteria that are necessary to survival of bivalve larvae, or both.

To understand bacillary necrosis in a more holistic manner, it is necessary to complement culturable with non-culturable microbial data. While culturable techniques are invaluable for studies of larval-pathogen interactions, nutrient media used to grow bacteria has been shown to only detect a fraction of bacterial communities in seawater (Powell et al., 2013) and bivalve (Romero and Espejo, 2001) samples. Most information on bacillary necrosis to date are based on culturable data and it could be primarily for this reason that we understand very little about bacillary necrosis.

1.8 Thesis aims

The overall objective of this research is to improve our understanding of the bacterial disease bacillary necrosis that affects commercial larval culture of the Australian blue mussel, *Mytilus galloprovincialis*. Currently there is very limited information on

bacterial pathogens of blue mussels and no studies have described the underlying microbial changes leading to bacillary necrosis.

The following specific aims of the thesis were to :

1) Examine how overfeeding of microalgae in model *M. galloprovincialis* larval cultures leads to rapid shifts in microbial communities and bacillary necrosis (Chapters 1 and 2). Experiments compared changes in culturable, with the focus of *Vibrio* (Chapter 1) and total bacterial communities (Chapter 2) in healthy and bacillary necrosis-affected larval cultures;

2) Examine how highly controlled seawater quality in larval culture can still lead to variability in occurrence and scale of mortality caused by bacillary necrosis (Chapter 3). Experiments compared shifts in culturable, total bacterial communities and correlations of elevated seawater proteolytic activity with mortalities and;

3) Examine how a novel bacteria isolated from a mass mortality event can negatively affect survival of reared Australian blue mussel. Experiments demonstrated virulence and characterised pathology.

Chapter 2 - Dynamic of culturable *Vibrio* abundance in early stage of blue mussel (*Mytilus galloprovincialis* Lamarck) larval rearing and associations with bacillary necrosis

2.1 Abstract

The culturable *Vibrio* population associated with blue mussel (*Mytilus galloprovincialis* Lamarck) larval rearing was examined during the first week post fertilisation to understand dynamics and contribution of culturable *Vibrio* to bacillary necrosis. Given the importance of organic matter in influencing *Vibrio* concentrations in hatchery environments, 3 replicate cultures were overfed with microalgae to examine if this induces a bacillary necrosis. Another 3 cultures were fed according to commercial standard and larvae were reared for 6 d, from 2 to 8 dpf. The overfed cultures attained the highest *Vibrio* concentration at $1.8 \pm 0.5 \times 10^5$ CFU ml^{-1} at 3 dpf, and decreased steadily by 1 log by 8 dpf. At 3 dpf (after 1 d of rearing), *Vibrio* represented >20 % of the seawater total viable count in 2 out of the 3 overfed cultures. Lower *Vibrio* concentrations were observed in the standard fed cultures ($1.9 \pm 1.0 \times 10^3$ CFU ml^{-1} at 3 dpf), decreasing to below detection after 4 d of rearing (at 5 dpf). The patterns of change in *Vibrio* relative abundance in seawater and larvae coincided with onset >30 % mortality and progressed to cumulative mortality of > 60 % by 8 dpf. *Vibrio* was not routinely detected in larvae samples except at the beginning of mortality at 377 ± 65 CFU $larva^{-1}$. Using larval challenge bioassays, dominant *Vibrio* and heterotrophic bacteria isolated from diseased larvae during

mortality demonstrated low virulence similar to that of *Vibrio* isolated from healthy commercial mussel hatchery larval cultures. The majority of *Vibrio* (11 of 12) were identified as members of the *Vibrio splendidus* group. This study suggests that *Vibrio splendidus* proliferation during mortality events result from growth of *Vibrio* communities normally associated with larval cultures.

2.2 Introduction

Australian blue mussel (*Mytilus galloprovincialis*) aquaculture is worth over $9.3 million with production of 3,500 t in 2011/2012 (Skirtun et al., 2013). Australia imports a further 35% of local production of fresh and frozen mussels whilst neighbouring New Zealand exported $180 million worth of farmed mussels in 2013 (Skirtun et al., 2013; Seafood New Zealand, 2014), indicative of high potential for increased local production. However, local mussel farming has been restricted by unreliable supplies of mussel spat mainly due to unpredictable and serious stock losses during the rearing of mussel larval and early age spat.

Members of the genus *Vibrio* have been frequently implicated as disease causative agents during larval rearing of many shellfish species. Unfortunately, information about the cause of mortalities in mussel hatcheries globally is very limited compared with other shellfish where mortality episodes have been well documented and are often shown to be due to bacterial causes. To date most detailed studies of bacterial pathogens of mussel are confined to the greenshell mussel (*Perna canaluculus*) larvae (Kesarcodi-Watson et al., 2009a; Kesarcodi-Watson et al., 2009b) which demonstrated *V. splendidus* and *V. coralliilyticus*-like bacteria are associated with the disease commonly referred to in shellfish hatcheries as bacillary necrosis.

However, Kwan and Bolch (2015) showed that substantial and genetically diverse *Vibrio* communities dominated by *V. splendidus*-related strains are also associated with healthy mussel larvae production. The abundance and association of *V. splendidus* related bacteria with diseased and healthy larvae indicates that that bacillary necrosis is not a single pathogen-host interaction, but a more complex interaction between the microbial community and the mussel larvae host.

Bacillary necrosis is a lethal disease in oyster (*Crassostrea gigas*) larvae and has close association with *Vibrio* (Tubiash et al., 1965; Tubiash et al., 1970). It has also been reported to affect larvae of various other molluscan species in marine hatcheries worldwide. In most cases, proliferation of opportunistic *Vibrio* to unusually high cell densities is a common characteristic even though the trigger of proliferation of causative agents are often complex phenomena influenced by host (Gomez-Leon et al., 2008; Chapman, 2012), environmental (Elston et al., 2008b; Kimes et al., 2012) and bacterial community factors (Connell et al., 1997; Gay et al., 2004b; Schulze et al., 2006). As more *Vibrio* species have been demonstrated to be causative agents of bacillary necrosis (Lodeiros et al., 1987), increases in *Vibrio* concentration in larval cultures can be serious and may indicate development of disease.

Studies showed *Vibrio* abundance in coastal seawater is closely correlated with algal growth (Thompson et al., 2004; Asplund et al., 2011) since algae represent a primary source of organic nutrients (Connell et al., 1997). Therefore, proliferation of *Vibrio* in larval cultures is not uncommon because larval cultures generally contain high levels of organic carbon derived from defecation, metabolite discharges and feeding activity of high biomass of larvae that can sustain *Vibrio* growth. This supports the

fact that *Vibrio* are ubiquitous in marine hatcheries (Sainz-Hernandez and Maeda-Martinez, 2005). High nutrient levels in larval cultures has been demonstrated to be an important factor of bacterial proliferation and can induce larval mortality (Connell et al., 1997; Eggermont et al., 2014).

Overfeeding of microalgae represents a poor husbandry practice because it further increases dissolved organic carbons (DOC) of larval cultures (Helm et al., 2004). Connell et al. (1997) demonstrated algal culture *Heterosigma carterae* and *Isochrysis galbana* generates high levels of DOC and can increase the concentration of *Vibrio* in oyster larval cultures. However, high *Vibrio* counts do not reliably predict mass mortality events indicating a more complex association between *Vibrio* and bacillary necrosis (Nicolas et al., 1996; Sugumar et al., 1998b; Sung et al., 2001; Chapman, 2012).

Occurrence of bacillary necrosis in a commercial Tasmanian blue mussel hatchery has been suspected based on occasional associations of larval death with bacterial counts on selective media thiosulfate-citrate-bile salts-sucrose (TCBS) and disease symptoms of cessation in swimming and feeding of larvae prior to severe level of mortality as also reported in studies (Jeffries, 1982; Garland et al., 1983; Lodeiros et al., 1987). The studies performed here aim to establish the contribution of culturable *Vibrio* to early-stage mussel larval mortality by examining: i) changes of *Vibrio* abundance in mussel larval culture during the first week; ii) association of *Vibrio* dominance with bacillary necrosis induced by overfeeding; and iii) genetic diversity and role of proliferated *Vibrio* in causing larval mortality using larval challenge bioassays.

2.3 Materials and methods

2.3.1 Larval cultures

Six larval cultures were established using 2 dpf "D" shaped blue mussel (*Mytilus galloprovincialis*) larvae sourced from Spring Bay Seafoods (SBS) commercial hatchery (Triabunna, Australia). The larvae were maintained in a fertilisation tank with no water exchange and no microalgal food added until collection and transport. For transportation, the larvae were concentrated on a 50 µm nylon mesh filter, wrapped in wet cloth and kept chilled in a polystyrene box during the 2 h transit. Transport of bivalve larvae wrapped in moist cloths and stored in cool condition is routine industry practice for transporting larvae. Post-transport survival is high even after overnight transport (Richards et al., 2015). Seawater for the larval culture experiment was filtered through a 1 µm bag filter and UV treated at the hatchery, collected in 20 l drums and transported to the laboratory. In the laboratory larvae were transferred into a 20 l bucket with gentle aeration before being homogenised by gently mixing and allocation into the larval culture containers. Each culture unit consisted of a 500 ml conical flask containing 5,000 mussel larvae with culture density of 10 larvae ml^{-1}. All larval cultures were maintained at 22 °C, natural photoperiods and gentle aeration. At 2 dpf onwards, the larvae were fed axenic *Isochrysis* sp. (*T. iso* clone) and the algal concentrations were determined using a haemocytometer. In order to induce bacillary necrosis, 3 larval cultures (H1, H2 and H3) were intentionally overfed with 100,000 algal cells ml^{-1} whilst 3 cultures S4, S5 and S6 were maintained at optimal commercial food concentration of 60,000 algal

cells ml^{-1}. Algal cells were replenished after a total water exchange every 2 d using seawater collected from the hatchery.

The larvae were reared from 2 to 8 dpf. Daily mortality was determined by direct examination using a dissecting microscope (Leica MZ12.5, 40 and 100 ×, Germany) from triplicate 5 ml sub-samples (containing approximately 50 larvae). Larvae were considered dead if there was an absence of shell content, occurrence of body/shell disintegration, or larvae exhibited no swimming or ciliary activity and no internal organ movement.

2.3.2 Bacterial plate counts of seawater and larvae sample

Seawater and larvae were sampled from each larval culture on a daily basis and prior to water exchanges and addition of algal feed. Cultures were mixed by increasing aeration to obtain homogenous larval distribution. Using a sterile syringe, 5 ml of seawater containing larvae was gently removed and pre-filtered through an autoclaved 50 µm mesh (Allied Filter Fabrics Pty Ltd., Hornsby, NSW, Australia) to separate larvae from seawater, and the seawater was collected into a sterile sample jar and vigorously vortexed. Approximately 50 larvae collected on the mesh were rinsed 3 times with autoclaved seawater and homogenised in 1.5 ml centrifuge tubes using a micro-pestle with addition of 0.5 ml of sterile seawater. Sub-samples of mixed seawater and larvae homogenates were serially diluted from 10^{-1} to 10^{-6} in autoclaved seawater. All dilutions were plated using an Advanced Instruments Autoplate® 4000 Spiral Plater (Advanced Instrument, Inc., Norwood, United States). For a single dilution series, two plates of Marine Agar (MA, Zobell, 1944) and thiosulfate-citrate-bile salts-sucrose (TCBS, Oxoid) agar plates with 30-300

colonies after 48 h of incubation at 23 °C were counted and averaged to determine colony forming units (CFU) per ml of seawater and per larva.

2.3.3 Bacterial characterisation using *atp*A and 16S rRNA gene sequences

Bacterial colonies from larval sub-samples during mortality onset were randomly selected and sub-cultured from the highest dilution TCBS (12 colonies) and MA plates (9 colonies). Colonies were re-streaked to obtain pure cultures and incubated for 48 h. Single bacterial colonies were picked using a sterile wooden toothpick and used as DNA templates for PCR. Each colony was resuspended in 200 µl of MQ water in a sterile 1.5 ml centrifuge tube, the tube vortexed until the colony was completely dispersed, and the cell suspension stored frozen at -20 °C until used in PCR. The cell suspensions were sufficient as PCR template without further processing. Presumptive *Vibrio* strains were identified by sequencing and comparative analysis of the bacterial ATPase (*atp*A) alpha subunit gene as it has been demonstrated to have sufficient phylogenetic resolution to distinguish intra-specific diversity of *Vibrio* (Thompson et al., 2007; Kwan and Bolch, 2015) and is a cheaper and sufficient alternative to Multilocus Sequence Typing (MLST). Degenerate PCR primers for the *atp*A gene were used (Thompson et al., 2007) (*atp*A37F,5'-CTDAATTCHACNGAAATYAGYG-'3; *atp*A1554R, 5'-TTACCARGWYTGGGTTGC-'3). The thermal cycling program consisted of i) 96 °C for 10 min, ii) 30 repetition of 95 °C for 1 min, 49 °C 1 min and 72 °C 2 min, and iii) final extension at 72 °C for 10 min. Bacterial colonies isolated and picked from MA plates were identified by PCR amplification of the 16S rRNA gene using primer

27F, 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-TACGGYTACCTTGTTACGACTT-3' (Weisberg et al., 1991). PCR reactions for both target genes were carried out in 25 µl consisted of 12.5 µl Red Immomix (Bioline, UK), 2 µl of bacterial cell suspension to provide DNA templates, 1 µl of 10 µM of each forward and reverse primers, and 8.5 µl of double-distilled water.The thermal cycling program used consisted of i) 96 °C for 10 min, ii) 30 repetitions of 95 °C for 1 min, 58 °C 1 min and 72 °C 2 min, and iii) final extension at 72 °C for 10 min.

PCR amplicons were analysed by agarose electrophoresis using 1 % (wt/vol.) agarose gels stained with GelRed (Biotium, USA) and visualised under UV light. PCR products of the *atp*A and 16S rRNA genes were examined for the expected target size (each approx. 1,500 bp). Successful PCR amplicons were purified using a MoBio UltraClean PCR Kit (MoBio, USA). The distal portion of the purified 16S rRNA and *atp*A gene were sequenced from the reverse amplification primer, using Big-Dye terminator sequencing chemistry (Applied Biosystems, USA) carried out at The Ramaciotti Centre for Genomics, University of New South Wales (Sydney, Australia). Raw sequence data were viewed and manually corrected using Geneious v5.4.6 (Biomatters, New Zealand) software. Corrected sequences were used as queries for sequence matching and nearest-neighbour comparisons using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/).

Phylogenetic relationships of 11 *Vibrio splendidus* related isolates were compared using 6 type strains and 34 reference *Vibrio* strains from the NCBI database, and 38 *Vibrio* genotypes from a previous study of hatchery-reared mussels showing no

disease symptoms (Kwan and Bolch, 2015). The phylogenetic tree was constructed by Neighbour-Joining based on Tamura-Nei genetic distances. Bootstrap analysis was performed using 500 replicates.

2.3.4 Bacteria-larval challenge bioassays

The virulence of the total 21 bacteria isolated from larval samples was determined using a mussel larval challenge bioassay. Mussel larvae for assays were obtained by thermally induced spawning of matured female and male hatchery broodstock and reared in 1 µm filtered seawater maintained at 22-23 °C and fed with *Isochrysis* sp. (*T. iso* clone) until 5 dpf. For comparison purpose, 27 of the 38 *Vibrio* genotypes isolated from the apparently heathy hatchery-reared mussels (Kwan and Bolch, 2015) were included for bioassay challenge. Bacterial isolates were cultured in Marine Broth (MB, Difco) for 36 h at 25 °C with constant shaking at 150 rpm. A volume of 0.8 ml of bacterial suspension was transferred into 1.5 ml centrifuge tube and centrifuged at 3,200 x *g* for 10 min. The supernatants were discarded and pelleted cells were re-suspended and washed twice in 0.8 ml of autoclaved sterile seawater. Larval challenge bioassays were carried out in 96 well plates with each well containing 40 to 50 animals in 200 µl of 1 µm filtered seawater with no algal food added. Bacterial suspensions were aliquoted to achieve a challenge concentration of 10^7 CFUml^{-1} that was confirmed by plate counts. The bacterial-larval challenge bioassays were carried out once with each bacterial isolate tested in triplicate wells. A highly virulent strain of *Vibrio tubiashii* characterised by larval immersion bioassay according to criteria of Mittal et al. (1980) was obtained from the Animal Health Laboratories (Isolate ID: 09/2885-1; at 10^7 CFUml^{-1}) and included in all

assays as positive control. Negative controls received sterile seawater. Immotility and mortality was examined at 2 and 6 day post challenge (dpc) respectively using a dissecting microscope (Leica MZ12.5, × 40 and × 100 magnification, Germany). Non-motile larvae showing ciliary beating and intestinal movement were classed as immotile. Larvae were classed as dead when neither swimming, cilliary activity nor intestinal activity was observed. After the assessment, the sampled larvae (both live and dead) were returned to the bioassay wells.

2.3.5 Data analysis

Bacterial abundance figures were normalised using log transformation. Comparisons of bacterial abundance in overfed and standard fed larval cultures during the 6 culture days were performed using the general linear model repeated measures procedure (p=0.05). For larval challenge bioassays, percent mortality figures were normalized using arc sine square root transformation. Mortality mean comparisons were carried out using one-way ANOVA (p=0.05). Post hoc comparisons between mortality levels were achieved using Tukey's range tests. All statistical analysis were carried out using SAS 9.4 (SAS Institute, Cary NC).

2.4 Results

2.4.1 Seawater bacterial counts

Bacteria were consistently more abundant ($5.3 \times 10^4 \pm 4 \times 10^4$ to $2.3 \times 10^6 \pm 0.7 \times 10^6$ TVC cells ml^{-1} in overfed cultures compared to standard fed larval cultures ($F=31.86$, df 1, 4, $p<0.001$). A consistent pattern of decrease in bacterial concentration after 3 d was evident in all larval cultures regardless of feeding

level, however the decrease did not clearly coincide with water changes on days 4, 6 and 8 (Figure 2-1). Culturable *Vibrio* in seawater were similarly more abundant in overfed cultures ($F=225.95$, df 1, 4, $p<0.001$), and steadily decreased in concentration in both optimally fed and overfed cultures (Figure 2-1). Overfed cultures started at $1.8 \pm 0.5 \times 10^5$ CFU ml^{-1} and decreased steadily to $1.4 \pm 0.1 \times 10^3$ CFU ml^{-1}, representing a 132-fold decrease in abundance. Similarly, optimally fed cultures started at $1.9 \pm 1.0 \times 10^3$ CFU ml^{-1} and decreased to below detection after 2 d of rearing.

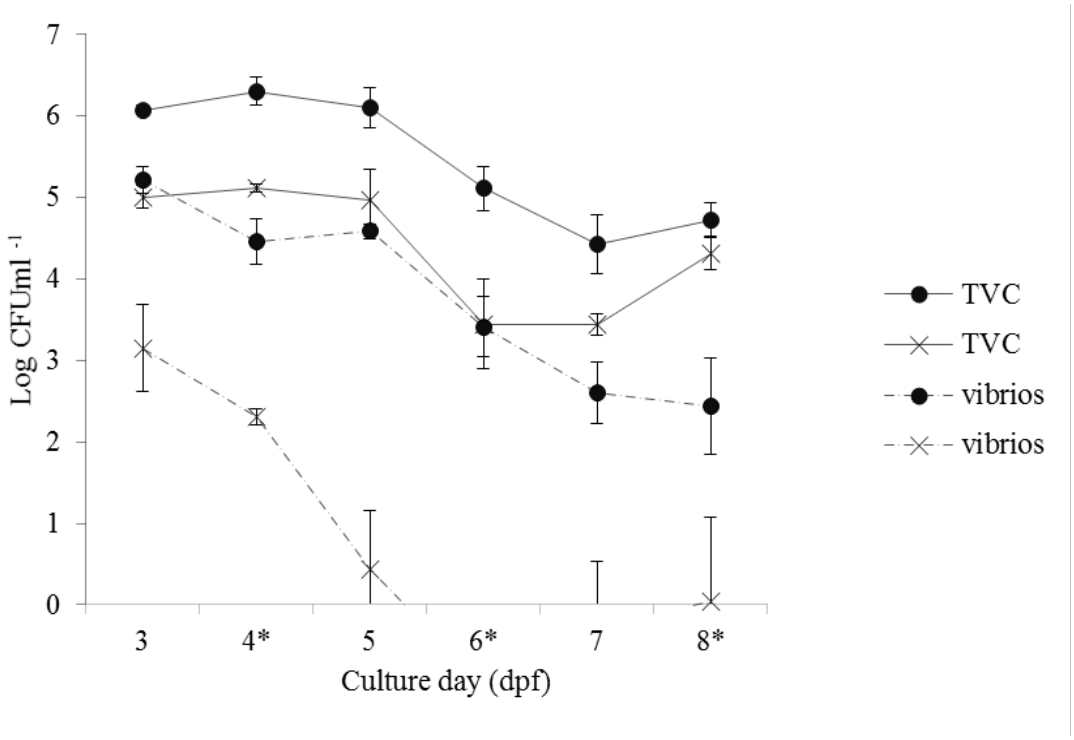


Figure 2-1 Mean total viable count (TVC) and presumptive culturable *Vibrio* in seawater of overfed (●) and standard (×) fed mussel larval cultures reared from 2 to 8 dpf. Bar indicates standard errors for 3 larval culture replicates. Solid and dotted line representing TVC and *Vibrio* respectively. * denote total water changes and replenishment of algal cells.

2.4.2 Larval bacterial counts

The pattern of bacterial abundance over time was more variable in overfed larvae, varying up to 2 orders of magnitude over the experiment and therefore was not averaged (Figure 2-2). Overfed larvae contained approximately 9.5 times more bacteria compared with optimally fed larvae ($F=11.11$, df 1, 4, $p<0.001$). The highest total viable counts were detected in overfed culture H1 at 4 dpf, and H3 at 5 dpf recording 3.6×10^4 CFU larva^{-1} and 5.1×10^4 CFU larva^{-1} respectively. Overfed cultures H1 and H3 contained detectable *Vibrio* at 4 and 5 dpf respectively (averaging 2.8×10^2 and 4.7×10^2 CFU larva^{-1}, respectively). *Vibrio* were not detected in larvae at other days.

Larval mortalities were associated with bacterial abundance (TVC and *Vibrio*s) in mussel larvae compared with those of seawater. The sudden appearance of larval mortalities in H1 and H3 at 4 and 5 dpf coincided with highest TVCs at 3.5×10^4 and 5.0×10^4 CFU larva^{-1} respectively (Figure 2-2). In contrast, the apparently normal mussel larvae showed no TVCs higher than 2.6×10^3 CFU larva^{-1}. The increase in *Vibrio* concentration in larvae during times of mortalities in H1 and H3 (280 and 474 CFU larva^{-1}) were unusual as TCBS did not detect *Vibrio* in larvae samples from other times (Figure 2-2).

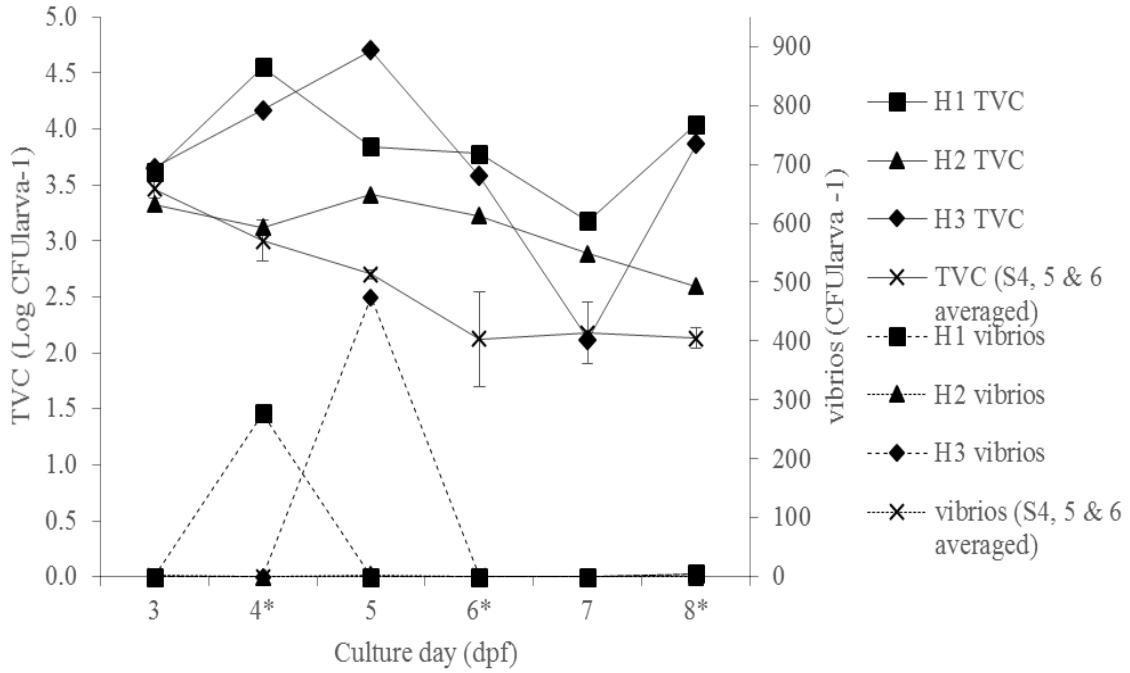


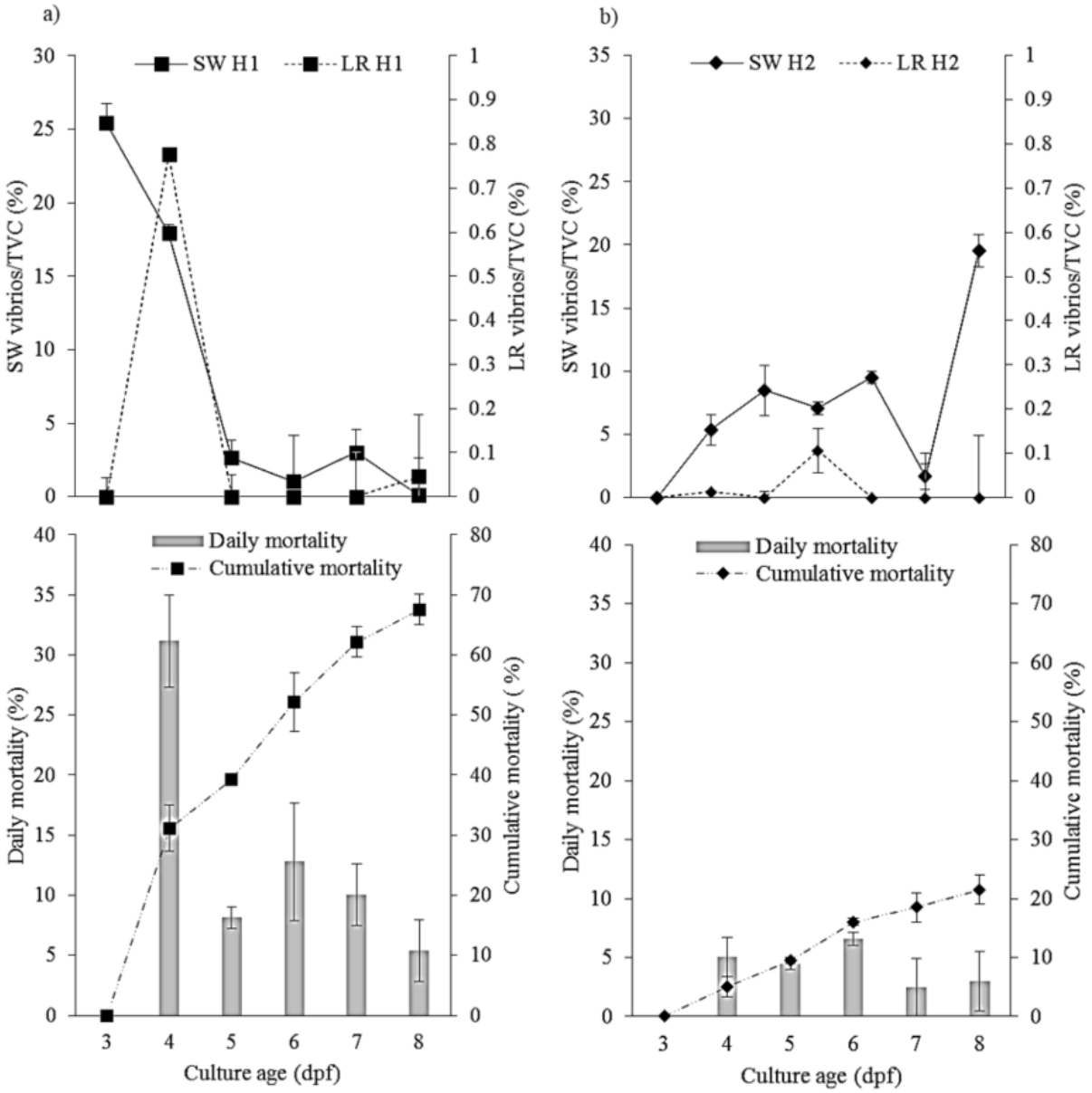
Figure 2-2 Total viable count (TVC) and presumptive *Vibrio* in mussel larvae of individual overfed culture H1 (■), H2 (▲) and H3 (♦); and 3 standard fed cultures (i.e S4, S5 and S6) averaged (×). Larval cultures were reared from 2 to 8 dpf. Bar indicates standard errors for 3 standard fed larval culture. Solid and dotted line representing TVC and *Vibrio* respectively. * denote total water changes and replenishment of algal cells.

2.4.3 *Vibrio* relative abundance and larval mortality

All 3 standard fed larvae cultures exhibited low mortality (mean of < 5 % per d) till 5 dpf, then steadily increased to a cumulative mortality of less than 15 % by 8 dpf in all cases. Mortality in overfed larval cultures H1 and H3 increased to > 30 % after 4 and 5 dpf respectively, with elevated mortality occurring despite water changes. Cumulative mortality reached approximately 65 % by 8 dpf. Mortality was lower in overfed culture H2, increasing steadily from 4 dpf to 21.5 % by 8 dpf.

A consistent pattern of increased *Vibrio* relative abundance was observed in seawater and then larvae in cultures experiencing elevated mortality. Mortality responses usually followed within 24-48 h by increased *Vibrio* relative abundance in larvae and simultaneous reduction in seawater *Vibrio* relative abundance (Fig. 2-3a & c). In H1, seawater *Vibrio* showed higher relative abundances at 25 % at 3 dpf but rapidly decrease by 5 fold to less than 5 % within 2 d. The reduced relative abundance in seawater *Vibrio* coincided with a rise in larval *Vibrio* although abundance was less than 1 % (Figure 2-3a).This sequence of events is similar in overfed culture H3 (Figure 2-3c) but was not observed in H2 (Figure 2-3b) or in any of the 3 standard fed cultures (Figure 2-3d) that otherwise showed good survival.

Larval mortalities were also clearly associated with bacterial abundance (TVC and *Vibrio*s) in mussel larvae compared with those of seawater. The sudden appearance of larval mortalities in H1 and H3 at 4 and 5 dpf coincided with highest TVCs at 3.5 x 10^4 and 5.0 x 10^4 CFU $larva^{-1}$ respectively (Figure 2-2). In contrast, the apparently normal mussel larvae showed no TVCs higher than 2.6 x 10^3 CFU $larva^{-1}$. The increase in *Vibrio* concentration in larvae during times of mortalities in H1 and H3 (280 and 474 CFU $larva^{-1}$) were unusual as TCBS did not detect *Vibrio* in larvae samples from other times (Figure 2-2).


a)
SW H1
LR H1
SW vibrios/TVC (%)
LR vibrios/TVC (%)
Daily mortality
Cumulative mortality
Daily mortality (%)
Cumulative mortality (%)
Culture age (dpf)
b)
SW H2
LR H2
SW vibrios/TVC (%)
LR vibrios/TVC (%)
Daily mortality
Cumulative mortality
Daily mortality (%)
Cumulative mortality (%)
Culture age (dpf)

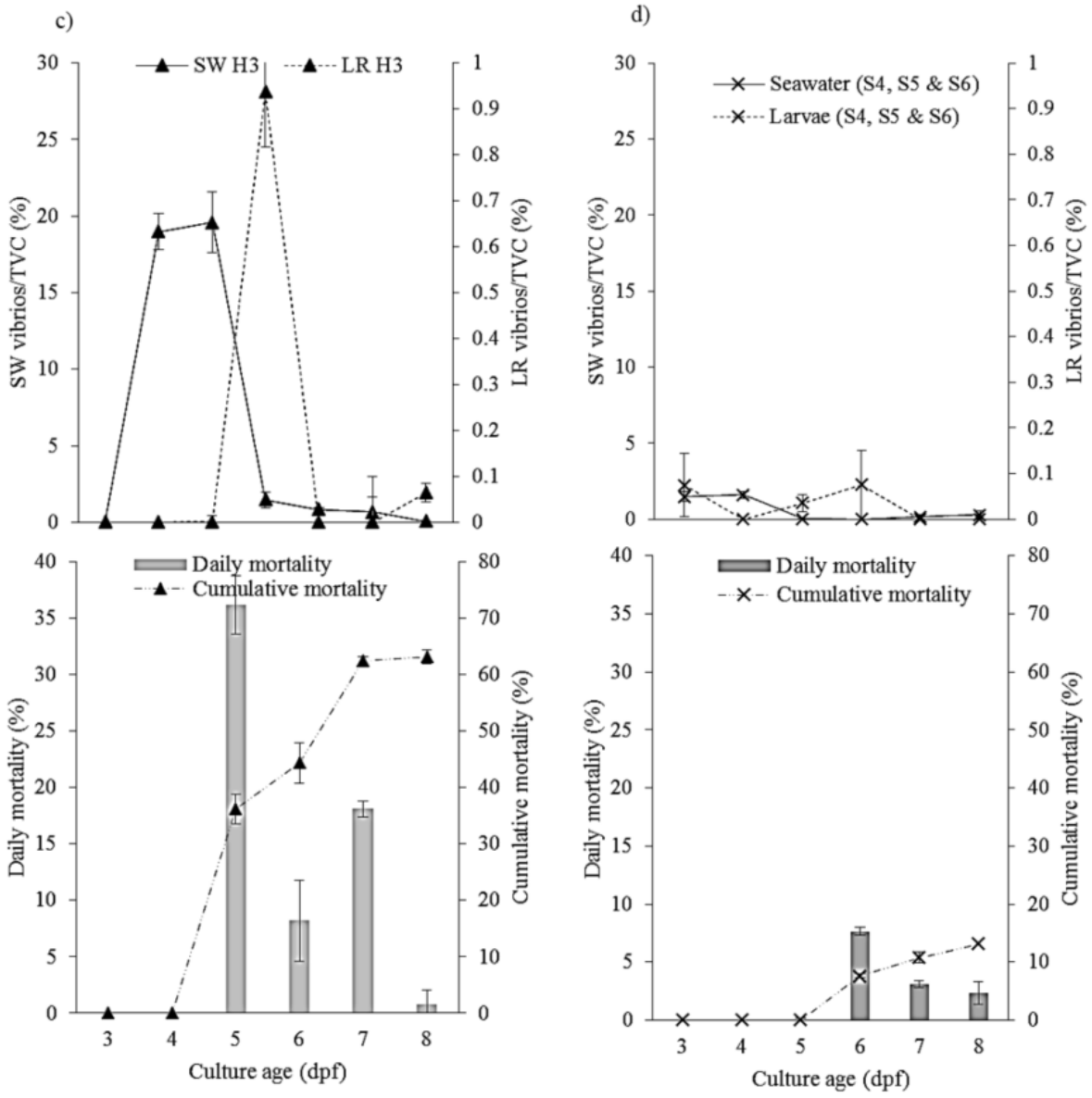


Figure 2-3 Overlay of relative abundance of presumptive *Vibrio* of individual overfed culture a) H1 (■), b) H2 (♦), c) H3 (▲) and d) averaged of 3 standard fed culture (i.e S4, 5 and 6) (×) with respective mortality of larval cultures reared from 2 to 8 dpf. The solid and dotted line represents *Vibrio*s/TVC (%) associated with seawater (SW) and larvae (LR) respectively. The histogram and broken line represents mortality (%) occurred at each day (i.e daily mortality) and cumulatively. Error bar indicates standard error.

Table 2-1 Genetic affiliation of *Vibrio* (V) and heterotrophic bacteria (H) isolated from diseased mussel larvae sampled during onset of mass mortality in overfed cultures H1 and H3 at 4 and 5 dpf respectively. Affiliation were based on partial sequence of the alpha subunit of bacterial ATPase (*atp*A) and 16S rRNA. Phylogenetic group of these *Vibrio splendidus*-like isolates were described in Figure 2-4.

				Similarity	Affiliation
Isolate	Source	Gene	Size (bp)	GenBank (accession no.)	*V. splendidus* clade (Kwan and Bolch, 2015)
V6, V21, V24	H3, 5dpf, larvae	*atp*A	869	100%, *Vibrio cyclitrophicus* strain FALZ170 (GU378434.1)	Group A-*V. cyclitrophicus* related
V16, V18, V26, V27, V28	H3, 5dpf, larvae	*atp*A	869	99%, *Vibrio splendidus* strain W10_1636/1 (KJ423058.1)	Group B-*V. celticus* related
V20	H1, 4dpf, larvae	*atp*A	869	99%, *Vibrio splendidus* strain W10_1636/1 (KJ423058.1)	Group C-*V. atlanticus* and *tasmaniensis* related
V14, V17,	H1, 4dpf and H3, 5 dpf larvae	*atp*A	869	99%, *Vibrio splendidus* strain R14789 (EF601289.1)	Group D-*V. lentus* related
V15	H1, 4dpf, larvae	*atp*A	769	90%, *Vibrio* genomosp. F10 strain FALZ129 (GU378421.1)	
H1	H1, 4dpf, larvae	16S rRNA	745	97%, *Alteromonas stellipolaris* strain PQQ-44 (CP015346.1)	
H3	H1, 4dpf, larvae	16S rRNA	774	99%, *Alteromonas stellipolaris* strain PQQ-42 (CP015345.1)	
H38	H3, 5dpf, larvae	16S rRNA	538	96%, *Alteromonas* sp. H2-71 (KM979192.1)	
H2	H1, 4dpf, larvae	16S rRNA	749	98%, *Pseudoalteromonas* sp. AG10 (KT121437.1)	
H31	H3, 5dpf, larvae	16S rRNA	841	99%, *Pseudoalteromonas* sp. JXH-45 (KR012043.1)	
H12	H1, 4dpf, larvae	16S rRNA	807	99%, *Olleya* sp. R18-12 (KT449868.1)	
H4, H30	H1, 4dpf,H3, 5 dpf larvae	16S rRNA	704, 761	97%, *Sulfitobacter* sp. S7-80 (KU999998.1)	
H11	H1, 4dpf, larvae	16S rRNA	744	98%, *Sulfitobacter dubius* strain PC (KX218293.1)	
H42	H3, 5dpf, larvae	16S rRNA	Poor sequences		

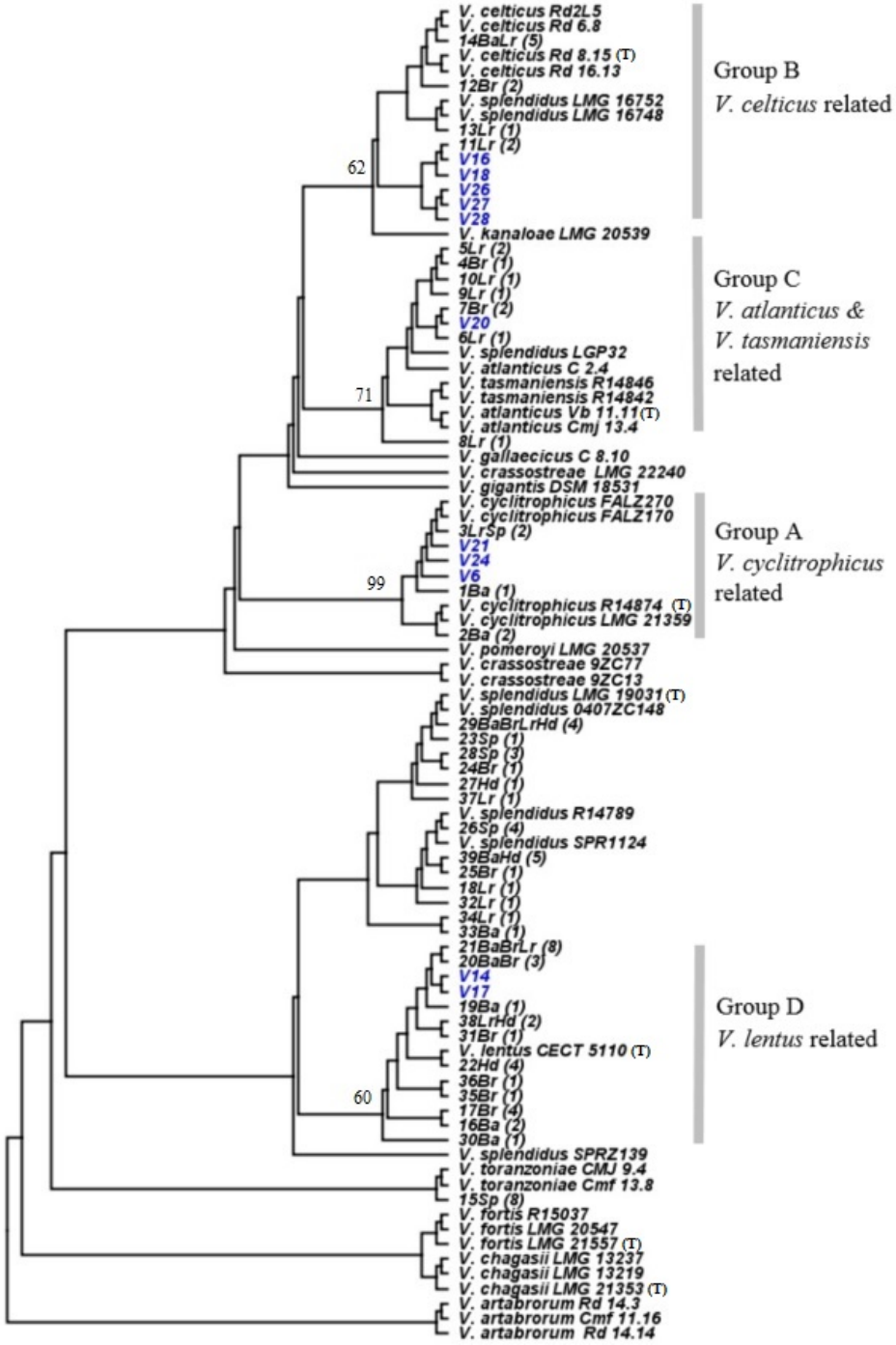


Figure 2-4. Phylogenetic relationships of 11 *Vibrio* isolates (based on 869 bp of *atp*A gene sequence) belonged to the *Vibrio splendidus* group isolated from mussel larvae during onset of mortality in overfed cultures H1 and H3 at 4 and 5 day postdpf respectively. The isolates (in blue) affiliated with different genotypic groups (Group A to D) established based on a study to examine genetic diversity of *Vibrio splendidus* associated with a commercial mussel hatchery (Kwan and Bolch, 2015). Tree was constructed by neighbour-joining from Tamura-Nei genetic distances. All 4 groups containing the 11 isolates show >50% bootstrap support (500 replicates). Isolate V15 (*Vibrio* genomosp like isolate) was not included in the tree. Type strains are marked with (T).

2.4.4 Culturable *Vibrio* associated with increased mortality

The *atp*A sequence data show that 11 out of 12 *Vibrio* isolated during proliferation of *Vibrio* in diseased mussel larvae belonged to the *V. splendidus* group (Table 2-1). Phylogenetic examination affiliated the 11 isolates to the species *V. celticus, V. cyclitrophicus, V. lentus, V.* tasmaniensis, and *V. atlanticus* (Figure 2-4). Isolate V15 was found to be most closely affiliated with *Vibrio* genomosp. F10 strain FALZ129 (GU378421.1) at maximum sequence homology of 90 %. Based on 16S rRNA gene sequences, 5 out of 9 bacteria isolated from diseased larvae were members of class *Gammaproteobacteria*, 3 of which belonged to the genus *Alteromonas* while the 2 others belonged to *Pseudoalteromonas*. Four of the 9 isolates were allied with the genera *Sulfitobacter* (class *Alphaproteobacteria*) and *Olleya* (class *Flavobacteriia*).

2.4.5 Virulence of bacteria isolates associated with mussel larvae mortality

At 6 dpc, 9 of the total 22 isolates did not produce mortality levels different to negative control (Figure 2-5) despite high challenge concentrations of 10^7 CFUml^{-1} in 1 µm filtered seawater and after prolonged durations. Thirteen of the total isolates produced mortality levels higher (p<0.05) than those observe in the negative control. Pairwise Tukey's range tests indicated isolate V16 and V18 produced highest mortality at 49 %, though only half the mortality observed in bioassay inoculated with *Vibrio tubiashii* positive controls. Interestingly, these two isolates were genetically similar based on *atp*A gene sequences (Figure 2-4) and positioned in the genetic group including *V. celticus* and *V. splendidus.* Isolates V6, V17 and V26 affiliated with *V. cyclitrophicus, V. lentus* and *V. celticus* respectively produced 2

times higher mortality (over 40 % mortality) compared with the negative control. Only 3 isolates from from MA agar, showed significantly higher mortality (over 30 %) than seawater controls; two belonged to the genus *Alteromonas*. Overall, the bacteria isolated during episodes of high mortality showed low virulence in assays and progress of mortality (over 6 d) was much slower than that observed in overfed larval cultures H1 and H3 at 4 and 5 dpf (Figure 2-3).

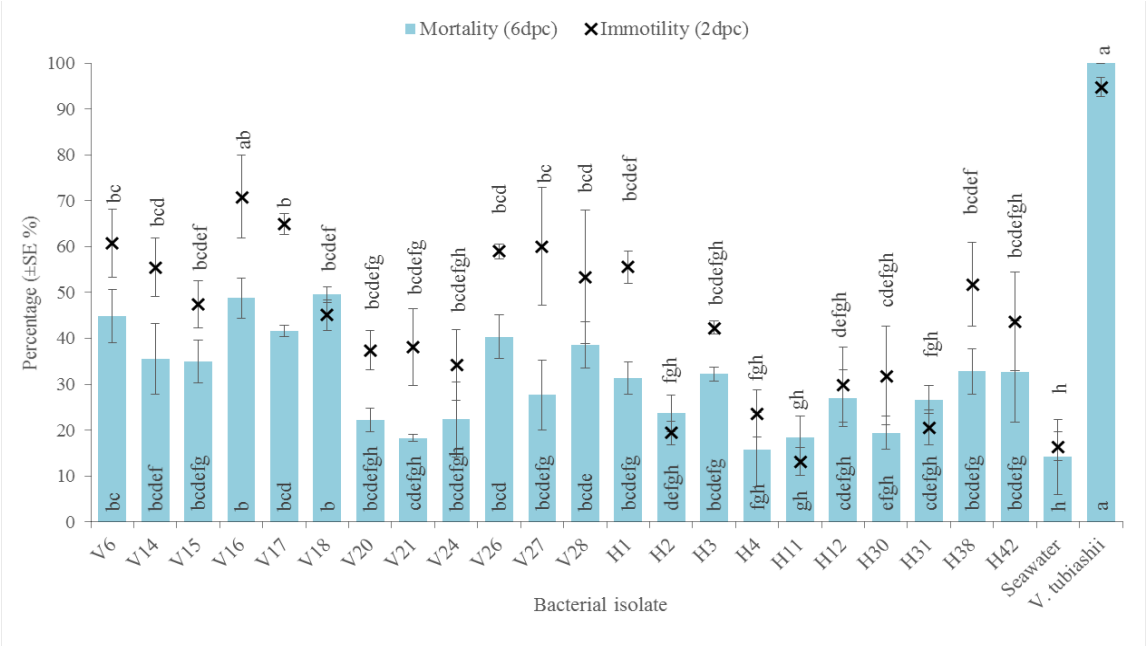


Figure 2-5 Mean percentage (± SE) of immotile mussel larvae at 2 dpc(represented as × plot), and cumulative mortality at 6 dpc (histogram) in larval-challenge bioassay inoculated with bacteria isolated from diseased mussel larvae. Statistical significant pairwise comparisons of immotility and mortality are marked with different letters (p<0.05). Bioassays were carried out at challenge concentration 10^{7} $CFUml^{-1}$ in triplicate, each used 40 to 50 five dpf larvae in 200 µl of 1 µm filtered seawater. Positive control received oyster pathogen *Vibrio tubiashii* (Isolate ID: 09/2885-1). Negative control received sterile seawater.

Twenty *Vibrio* isolates produced significantly higher mortality (average of 43 % at 6 dpc), than seawater controls (F=11.86, df=24, 50, p<0.05). Mortality > 45 % was shown by isolates V77 and V78 that belonged to *V. tasmaniensis* and *atlanticus* group, and V14, V17, V41, V49 and V70 which belonged to the *V. splendidus* and *V. lentus* group.

Increasing mortality was gradual in both *Vibrio* isolated from the diseased larvae (present study) and healthy larvae (previous study). The level of mortality at 6 dpc correlated with larval motility at 2 dpc (see Appendix 2). All *Vibrio* examined in the bioassays negatively affected the swimming activities of larvae (compare Figure 2-5 and Figure 2-6) resulting in up to 70 % immotile larvae. *Vibrio* isolated from mortality events in the present study resulted in an average of 54 % immotile larvae, comparable to the immotility caused by *Vibrio* isolates from healthy larvae (average 53 %). Immotile larvae often aggregated at bottom of challenge well (see Figure 2-7) and showed frequent retraction of the velum. Close examination of immotile larvae showed activity of internal organs comparable to that of healthy individuals.

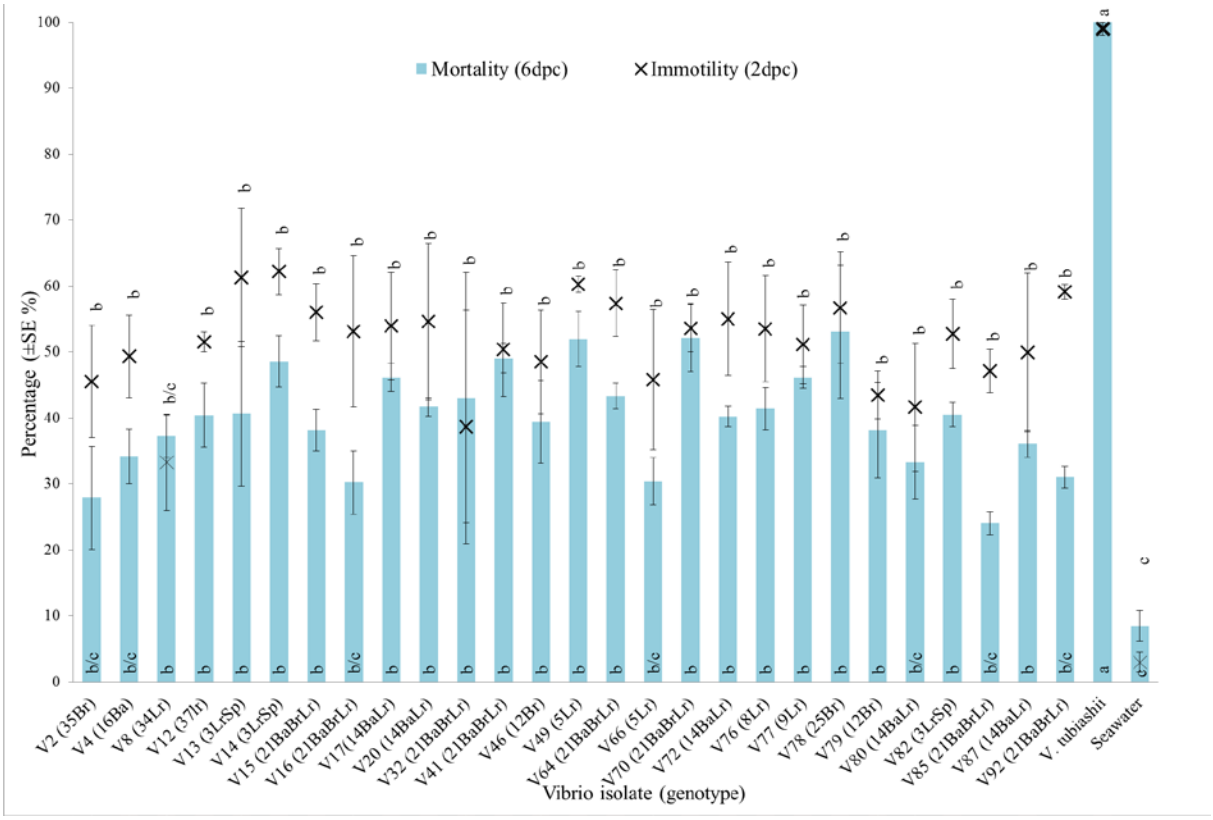


Figure 2-6 Mean percentage (± SE) of immotile mussel larvae at 2 dpc (represented as × plot), and cumulative mortality at 6 dpc (histogram) in larval-challenge bioassay inoculated with *Vibrio splendidus* group strains isolated from apparently disease free blue mussel cultures in a commercial hatchery (Kwan and Bolch, 2015). Statistical significant pairwise comparisons of immotility and mortality are marked with different letters (p<0.05). Bioassays were carried out at challenge concentration 10^{7} $CFUml^{-1}$ in triplicate, each used 40 to 50 five dpf larvae in 200 µl of 1 µm filtered seawater. Positive control received oyster pathogen *Vibrio tubiashii* (Isolate ID: 09/2885-1). Negative control received sterile seawater.

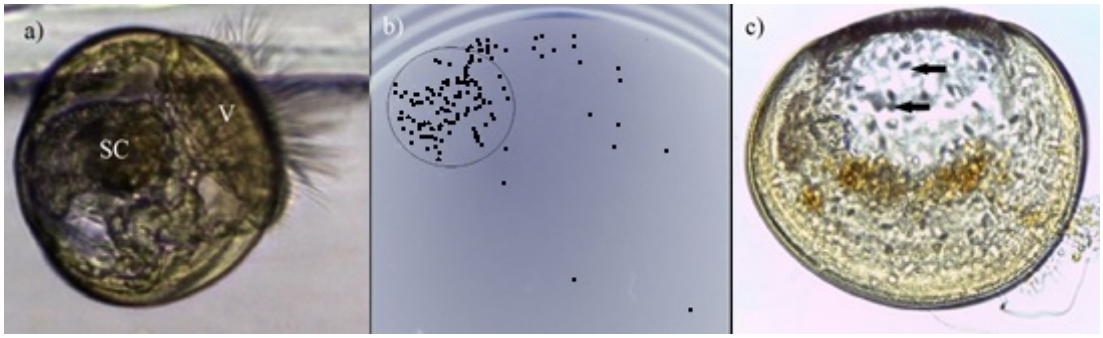


Figure 2-7 a) Healthy mussel larvae (5 dpf) prior to larval challenge bioassay showing shell contents (SC) and velum (V) b) immotile larvae (individual larvae darken to aid visibility) aggregated at bottom of challenge well (circled) at 2 dpc c) dead larvae with intact but empty shell showing secondary attacks by ciliates (arrowed) at 6 dpc.

2.5 Discussion

Newly hatched larvae are vulnerable to infections by opportunistic bacteria due to their simple immune system (Ciacci et al., 2009). Therefore, larval cultures can be vulnerable to sporadic and unexplained mass mortality, causing a production bottleneck for mussel aquaculture (Kesarcodi-Watson et al., 2009a; Kwan and Bolch, 2015).

This study shows that overfed larval cultures support higher concentrations of bacteria (as TVC) and culturable *Vibrio* compared with standard fed cultures. Seawater and larvae were not sampled at the start of the experiment (2 dpf) therefore it is not clear whether the high *Vibrio* concentration at the start of larval culture was due to high *Vibrio* inoculum transferred with the larvae, or due to subsequent growth during the first 24 h of rearing. Plate-counting of microalgal feed for TVC and *Vibrio* using MA and TCBS did not detect any *Vibrio* colonies therefore ruling out feed as source of *Vibrio*. The larval cultures were established from the same batch of

larvae and seawater, yet plate counts show a 100-fold higher concentration of *Vibrio* in overfed cultures by 3 dpf, indicating that *Vibrio* abundance changed in the first 24 h. The higher concentrations of *Vibrio* in overfed cultures is likely to do with higher levels of DOM associated with higher feeding levels in the newly established larval culture. Connell et al. (1997) shows higher concentration of algal cells results in a higher concentration of DOM, and *Isochrysis galbana* culture contains DOM capable of stimulating *Vibrio* growth when used to feed oyster larvae. Therefore, the higher concentration of algal cells in the overfed cultures would lead to higher concentration of DOM supporting higher concentration of *Vibrio*. Oberbeckmann et al. (2012) found that *Vibrio* concentration in coastal seawater is positively correlated with algal bloom, further supporting the link of *Vibrio* proliferation with increased algal concertation.

Bacterial loads in marine larval rearing has been directly linked with DOM (Vadstein et al., 1993; Vadstein et al., 2004; Attramadal et al., 2012b) indicating nutrients as the typical determining factor. However, there were no clear cyclical changes in *Vibrio* abundance associated with regular water changes and algal food replenishment during the experiment (see Figure 2-1). Instead, *Vibrio* were most abundant at the beginning of the experiment and progressively decreased in abundance during rearing. This indicates that high organic matter alone is not sufficient to lead to increased *Vibrio*, suggesting a more complex mechanism regulating *Vibrio* populations in mussel larval cultures.

Vibrio proliferation at the beginning of the larval cultures but not in the later stages suggests transfer of mussel larvae from the fertilisation tank into the minimally

conditioned cultures plus the availability of DOM from microalgae represents a possible important trigger for *Vibrio* proliferation. The transfer of larvae into newly established cultures system introduces bacteria to unutilised resources where bacteria with versatile nutrient utilisation capabilities such as *Vibrio* can readily exploit available nutrients in the absence of competition, thus multiplying rapidly. Vermeer (2001) demonstrated freshly UV treated culture seawater with no *Vibrio* on TCBS plates attained counts of 1.2×10^4 CFU ml^{-1} within 24 h in larval scallop (*Agropecten irradians*) cultures. The higher *Vibrio* concentrations observed in the overfed cultures were greater than those reported by Vermeer (2001) and could be a response to the higher availability of organic carbon resulting from the increased algal food concentration.

The initially high *Vibrio* concentration in larval culture could also be due to high inoculum concentration of *Vibrio* associated with transfer of larvae from fertilisation tank. Broodstock as a source of *Vibrio* has been identified in many studies (Lodeiros et al., 1987; Sugumar et al., 1998b). Sainz-Hernandez and Maeda-Martinez (2005) observed that spawning activities of scallop broodstock resulted in substantial *Vibrio* blooms within a period of 24 h. In this study, larvae were transferred from fertilisation to culture without disinfection or vigorous rinsing therefore a substantial number of *Vibrio* may have been transferred with the larvae. The steady decrease of *Vibrio* cells could then be due to repeated water changes removing cells from culture system.

A progressive decrease in seawater *Vibrio* concentration regardless of feeding level is interesting. In this study, seawater used for rearing was collected post-UV

treatment in a commercial hatchery and stored in a 25 l drum for the larval rearing. The water may have gradually aged with storage and the lack of organic matter may limit growth of *Vibrio* in the replacement water. Aging of seawater, where seawater is held in a tank and allowed to settle for several days to over a week is often employed by marine hatcheries as a means to reduce incidences of mortality. Anecdotally, the aging process of seawater has potential to alter seawater bacterial communities by sedimentation of bacterial associated with particulate materials and deprivation of organic matter. This is possible because the majority of seawater bacteria are particulates-bound and removal of particulates generally reduces seawater bacterial abundance (Magnesen et al., 2013). Whittington et al. (2015) showed that simple physical treatment by aging seawater for 48 h is as effective as water filtration using mesh screen smaller than 5 µm in preventing oyster spat mortality caused by ostreid herpesvirus (OsHV-1 µVar), leading the authors to assume seawater aging helps remove viruses associated with particulates by sedimentation. The longer aging period of seawater for over 1 week in this study might have also caused similar sedimentation of *Vibrio* associated with particulates resulting in lower concentration in seawater used to replenish larval cultures. Given the potential benefits associated with seawater aging, future works can consider to understand how seawater aging affects bacterial communities and whether this can influence the outcomes of bacillary necrosis.

Temporary increases in relative abundance of *Vibrio* (referred to herein as *Vibrio* spikes) coinciding with increased mortality were observed in two of the three over-feed cultures. Similar *Vibrio* spikes have been observed in other rearing larval cultures of other shellfish species and occasionally coincide with mortality events

(Leibovitz and Elston, 1980; Jeffries, 1982; Lodeiros et al., 1987; Nicolas et al., 1996; Elston et al., 2008b; Genard et al., 2011). Similar phenomena have been observed for tropical rock lobster larval cultures (Webster et al., 2006). Culturable *Vibrio* were generally not detected in larvae samples except during periods of elevated mortality. The spikes of *Vibrio* relative abundance from below detection to over 280 and 474 CFU larva^{-1}at the times of mortality indicates that *Vibrio* spikes reflect larval heath and the observations are in agreement with other studies (Lodeiros et al., 1987; Sugumar et al., 1998b). However, there is limited information on the genetic diversity of *Vibrio* growth spikes. Based on the *atpA* gene as a phylogenetic marker, this study further shows that the growth spikes comprised mostly of diverse *Vibrio splendidus* related genotypes which otherwise would not be resolved using the 16S rRNA gene (Thompson et al., 2007; Kwan and Bolch, 2015).

A substantial body of literature has implicated *Vibrio splendidus* as causative of bacillary necrosis in shellfish larvae (Elston and Leibovitz, 1980; Lodeiros et al., 1992; Lambert et al., 1998; Paillard et al., 2004; Prado et al., 2014b; Richards et al., 2015). Whilst it is tempting to conclude that *Vibrio splendidus* growth spikes are a direct cause of larval mortality, neither the *Vibrio* nor any heterotrophic bacteria isolates assayed induced mortality at rates comparable to that observed in cultures H1 and H, despite the high cell concentrations and prolonged exposures used in the assays. The bioassays results indicate that these bacteria are unlikely to be the direct cause of larval mortality. The lack of virulence of the isolates from diseased larvae contradicts many studies which report the isolation of virulent *V. splendidus*-like bacteria from mass larval mortalities (such as Jeffries, 1982; Nicolas et al., 1996; Sugumar et al., 1998b; Kesarcodi-Watson et al., 2009a). This could be due to the

inability to isolate strains as a result of viable but non-culturable (VBNC) state (Armada et al., 2003), or that the *V. splendidus* group harbours a diverse range of genotypes (Kwan and Bolch, 2015) with variable virulence (Saulnier et al., 2010).

The use of single isolate challenge bioassay in this study may have prevented observation of inter-bacterial interactions to bring about disease. Pooled isolates bioassay by Gay et al. (2004a) observed synergistic effects of two *V. splendidus* isolates resulting in increased lethality when administered into adult oyster by injection. Similarly, Lemire et al. (2015) showed higher concentrations of a avirulent genotype of *V. splendidus* can increase the virulence of lower concentrations of a virulent genotype. The contribution of avirulent *V. splendidus* to disease has been shown to occur via genotype-independent quorum sensing. De Decker et al. (2013) demonstrated that high concentrations of avirulent *V. splendidus* produce and release auto-inducer compounds into the environment that can trigger expression of metalloprotease genes *vsm* and *vam*. These metalloproteases are a major virulence factor in both *V. splendidus* and *V. aestuarianus*. As a result, *Vibrio* spikes observed in this study may have more complex contributions to overall microbial community virulence and the next logical step of investigation would be to examine how these avirulent strains can facilitate disease by multi-strains bioassay challenges.

The larval bioassay challenges carried out here showed that some *Vibrio* affect the swimming activity of mussel larvae at high concentration (10^7 CFU ml^{-1}). Impaired swimming may have an indirect link to the deterioration and death of mussel larval. Several studies report behavioural changes preceding larval mortalities events, including loss of larval motility and cessation of feeding, resulting in larval

aggregation at the bottom of tanks (Disalvo et al., 1978; Elston and Leibovitz, 1980; Garland et al., 1983); *Vibrio* were implicated as the cause of these behaviours. Furthermore, Chapman (2012) observed that 6 out of 7 commercial oyster larval production that suffered mass mortality, were preceded by symptoms of larval aggregation and loss of larval motility.

Inhibition of filtration activity of mussels (*Mytilus edulis*) by *Vibrio* is common (Birkbeck and McHenery, 1982). Inhibitory effects of *Vibrio* on larval motility has also been reported and attributed to a low molecular weight toxin of 0.5 to 1 kDa produced by 85 % of pathogenic and 28 % of non-pathogenic *Vibrio* strains (Nottage and Birkbeck, 1987; Nottage et al., 1989). Larval motility was not examined during this experiment, therefore it is possible that the seawater *Vibrio* concentration in cultures in H1 and H3 (2.4×10^5 CFU ml^{-1}) were sufficient to affect motility during the first 24 h and facilitated the rapid development of disease in these treatments

2.6 Conclusion

The development of elevated *Vibrio* concentrations by 24 h after establishing in overfed larval cultures, followed by gradual decline suggest that *Vibrio* proliferation can be triggered by the interplay of microbial stability of newly established larval cultures and at the same time presence of higher levels of organic matter derived from increased algal feed input. However, the use of aged seawater used to replenish larval cultures is potentially low in *Vibrio* concentrations and could contribute to the progressive decrease in *Vibrio* concentrations despite availability of DOM. *Vibrio* spikes at early stage of larval cultures are closely associated with poor survival of larvae even though the study observed low virulence in the culturable *Vibrio* or

heterotrophic bacteria isolated from diseased mussel larvae during onset of mortalities. However, the majority of culturable *Vibrio* were members of the *V. splendidus* group that could have an indirect role in larval mortality by production of quorum sensing molecules triggering virulence other bacteria. This study suggests *Vibrio* proliferation in this case is likely outgrowth of normal *Vibrio* communities but could affect swimming activities of mussel larvae when present in high concentrations. This could indicate indirect links of proliferated *V. splendidus* bacteria to larval mortality as larval aggregation has been reported to always precede bacillary necrosis (Chapman, 2012). Overall, this study demonstrates *Vibrio* spikes are an important characteristic of bacillary necrosis but they have complex associations with larval mortality.

Chapter 3 Bacterial community dynamics associated with bacillary necrosis in Australian blue mussel (*Mytilus galloprovincialis* Lamarck) larval rearing induced by overfeeding

3.1 Abstract

Bacteria associated with the first week rearing of Australian blue mussel (*Mytilus galloprovincialis*) larvae in the overfed and standard fed larval cultures in Chapter 2 were further investigated using molecular techniques to determine bacterial communities associated with mass larval mortality induced by overfeeding. A total of 72 seawater and larvae samples (of which 6 associated with mass mortality) from overfed and standard fed larval cultures were analysed using Automated Ribosomal Intergenic Spacer Analysis (ARISA) of 16-23S rDNA intergenic spacer 1 (ITS1). The ARISA data showed that overfeeding resulted in significantly different seawater and larval associated bacterial communities when compared with standard fed cultures (PERMANOVA, seawater F=3.70, df 1, 34, p=0.001, and larvae F=2034, df 1, 34, p=0.011). Principal Coordinates (PCO) analysis of ARISA dataset showed mortality associated samples were separated from overfed culture samples that otherwise showed good survival. Canonical analysis of principal coordinates (CAP) catergorised samples (n=72) into healthy, a day before (n=2), during (n=2) and a day after mortality (n=2), demonstrated systematic shifts in both seawater and larval bacterial communities. These corresponded to the increase and subsequently decline in mortality levels. Changes in bacterial communities were observed up to 3 d

prior to onset of larval mortality with symptoms consistent with bacillary necrosis. Modified bacterial communities were observed in seawater prior to larval mortality and was followed within 24 h by similar changes in the larval microbial community, suggesting colonisation of larvae from the seawater community. High mortality level (>30 % per d) occurred only when both seawater and larval communities converged with a similar modified state of communities. Further examination of bacterial community composition using 454 pyrosequencing of 16S rRNA genes revealed that samples with elevated larval mortality showed increased dominance of strains related to *Psychroserpens mesophilus.* This study demonstrated for the first time the interplay between seawater and larval communities during bacillary necrosis associated with mussel larval rearing.

3.2 Introduction

Commercial hatchery production of blue mussels is relatively new compared to other bivalves species such as oysters, scallops, and clams, but is important for diversification of bivalve aquaculture (Sanchez-Lazo and Martinez-Pita, 2012). Hatchery production methods have been developed but larval rearing is affected by unexplained, sometimes severe mortality, particularly in the early larval and metamorphosis stages, resulting in increased production costs and inconsistent supply of mussel spat for adult grow-out (Anguiano-Beltran et al., 2004; Kesarcodi-Watson et al., 2009a).

The bacterial disease known as bacillary necrosis is suspected to affect hatchery reared mussel larva based on its typical characteristics of rapid mortality, often within 24-48 h, descriptions of similar pathological symptoms, such as cessation

of feeding, lipid depletion and abnormal swimming activity, and the fact that mussel larval mortality can be prevented by antibiotic treatments (Kesarcodi-Watson et al., 2009a; Kesarcodi-Watson et al., 2009b). Bacillary necrosis is of concern because it is widespread and has been documented to affect larval stage of various bivalve species such as oysters (Brown and Losee, 1978; Disalvo et al., 1978; Garland et al., 1983; Lodeiros et al., 1987; Prado et al., 2005), clams (Gomez-Leon et al., 2005; Antonio Guisande et al., 2008) and scallop larvae (Nicolas et al., 1996; Lambert et al., 1998; Jorquera et al., 2004), suggesting common disease mechanisms independent of host species.

Even though the majority of studies implicate marine bacteria, primarily opportunistic bacteria of class *Gammaproteobacteria* as the causative agents, the nature and underlying mechanisms of bacillary necrosis are still poorly understood due to its complexity and the lack of studies attempting to characterise the wider bacterial communities using a more powerful culturable independent techniques. This is necessary because culturable approaches provide a biased and incomplete picture of bacterial diversity and underestimates of bacterial populations. For example, Jorquera et al. (2004) shows plate counts represented less than 2 % of total bacteria in a scallop hatchery when compared with fluorescence based counting. Furthermore, recovery of bacteria on artificial media can be influenced by viable but non-culturable (VBNC) state of bacteria (Vezzulli et al., 2015).

Poor husbandry practices in larval cultures have been associated with bacillary necrosis (Brown and Losee, 1978). Elston (1993) recognised it as a hatchery management disease because of its opportunistic nature. So far, several studies

observed that bacillary necrosis has links to bacterial factors (Connell et al., 1997; Gay et al., 2004b; Schulze et al., 2006), which in some cases appeared to be triggered or exacerbated by rearing conditions. However, there is no study done to characterise response of bacterial communities to suboptimal rearing conditions and whether there are associations with bacillary necrosis.

Bivalve larval rearing creates an enriched and complex microbial environment because the stocking densities and conditions required for optimal growth also produces high bacterial loads (Garland et al., 1983). A major source of organic material in culture system is added larval food in the form of cultured microalgae. Hatcheries maintain strict control of food concentration through larval rearing and overfeeding is considered by hatchery operators to be a major risk factor for development of bacterial disease. For example, Eggermont et al. (2014) demonstrated stimulation of heterotrophic bacteria associated with healthy adult mussels (*Mytilus edulis*) by intentional addition of organic carbon in rearing seawater resulted in 80 % mortality in 3 d, which was preventable by addition of antibiotics. These observations are indicative of similar risks of high nutrients from overfeeding in causing mortality in mussel larval production.

The bacterial community associated with commercial blue mussel hatcheries is genetically diverse and varies considerably in different rearing systems and at different stages of larval rearing. Rapid shifts in predominant genotypes in the microbial community occur (Kwan and Bolch, 2015) and thus there is potential for rapid changes in virulence. Earlier study in Chapter 2 showed that overfeeding increased the risk of high mortality and *Vibrio* bacteria proliferated prior to and

during mortality events. However, it is unclear how overfeeding alters the wider larval and culture seawater communities and there is so far only limited culturable based evidence for a link between overfeeding and bacillary necrosis.

The first week post fertilisation larvae of the Australian blue mussel currently utilises static tank culture with water exchanges at 48 h intervals. Larvae are then transferred to flow through tanks till metamorphosis and settlement onto rope substrate. Sporadic and severe stock losses are reported during the first week of larval rearing and are not always associated with increased bacterial load. Given the lack of knowledge of bacterial disease in mussel hatcheries we sought to determine whether overfeeding resulted in consistent changes in the seawater and larval microbial communities and whether such changes were associated with elevated larval mortality during the first week of mussel larvae rearing.

3.3 Materials and Methods

3.3.1 Larval cultures

Experiments of Chapter 3 was based on the same larval cultures detailed in Chapter 2. Briefly, Australian blue mussel (*Mytilus galloprovincialis*) D-larvae (2 dpf) were supplied by Spring Bay Seafoods (SBS) commercial hatchery (Triabunna, Australia). Six 500 ml larval cultures were established in conical flasks at a culture density of 10 larvae ml^{-1}. Cultures were maintained at 22 °C in a temperature controlled room, using 1 µm filtered, UV treated 35 ppt seawater with gentle 0.2 µm filtered aeration and cultured for 6 d (2 to 8 dpf) under ambient lighting. Total water change and replenishment of feed using axenic *Isochrysis* sp. (*T. iso* clone) were carried out at 4

and 6 dpf (Table 3-1). Three larval cultures (S1, S2 and S3) were given a standard commercial feeding rate of 60,000 cells ml^{-1} (see Appendix 1 for feeding rate), and 3 cultures (H1, H2 and H3) received 100,000 cells ml^{-1}, a level considered overfed for Australian blue mussels. Daily mortality was determined by direct observation of subsamples of 50 larvae under a dissecting microscope (Leica MZ12.5, × 40 and × 100 magnification, Germany). Larvae were recorded as dead if they exhibited tissue disintegration, or no swimming, ciliary activity or internal organ movement could be detected.

3.3.2 Sampling and bacterial DNA extraction

Samples for DNA extractions were subsets of samples prepared in Chapter 2 for bacterial enumeration. Briefly, seawater and larvae were sampled daily prior to 100 % water changes (see Table 3-1 for experiment tasks order). Culture containers were gently mixed by aeration and a sterile syringe used to remove 5 ml of culture water (approximately 50 larvae). Samples were pre-filtered through autoclaved 50 µm mesh (Allied Filter Fabrics Pty Ltd., Hornsby, New South Wales, Australia) to separate larvae and the larvae transferred into autoclaved seawater for determination of mortality. Larvae were rinsed 3 times in autoclaved seawater, transferred to a 1.5 ml centrifuge tube with 0.5 ml of sterile seawater, and homogenised using sterile micro-pestles. Approximately 50 ml of culture seawater was poured through sterile 50 µm mesh to retain seawater fraction which was then filtered through sterile 0.22 µm Supor® 200 polyethersulfone PES membrane filters (Pall Corp.,New York, United States).

Table 3-1 Order of experimental routines for 6 larval culture units of Australian blue mussel using 48 h interval static cultures for the first week post fertilisation from 2 to 8 dpf.

	Culture day (dpf)						
Order of routine	2	3	4	5	6	7	8
1. Mortality assessment	×	√	√	√	√	√	√
2. Sample collection	×	√	√	√	√	√	√
3. Water and feed replacement	√	×	√	×	√	×	×

The filters and homogenised larvae were stored at -20 °C until extraction of DNA using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. The DNA digestion step used 2 × 1 cm^2 cut sections (representing 11.5 % of filter) and 100 µl of the larval homogenate. A total of 72 DNA samples (n=36 seawater; n=36 larvae) were collected from all six cultures at 6 time points, from 3 to 8 dpf inclusive, with each time point generating 2 sample types- seawater (S) and larvae (L). The 72 samples (for example, H1-3S) were identified by larval culture feeding treatment (i.e overfed culture H1), day of culture as dpf larvae as day post fertilisation (i.e 3), and type of sample (S for seawater).

3.3.3 Automated Ribosomal Intergenic Spacer Analysis (ARISA) of larval cultures bacterial community

Culture independent assessment of the microbial community was carried out using ARISA. The 16-23S rDNA intergenic spacer 1 (ITS1) region was PCR with universal bacterial primers 16S-1392F (5'- GYA CAC ACC GCC CGT-3') and 23S-125R (5'- GG GTT BCC CCA TTC RG-3') (Brown and Fuhrman, 2005). Each DNA sample was amplified in duplicate (i.e amplification reaction prepared and performed at different times) to minimise PCR biases. PCR reactions were carried out in 25 µl volume comprised of 12.5 µl Red Immomix (Bioline, UK), 1 µl of 10 µM 16S-

1392F, 1 µl of 10 µM 23S-125R, 1 µl of 1 mg ml^{-1} BSA, 2 µl of DNA extract as template. PCRs were performed in a C1000 Touch™ Thermal Cycler (Bio-Rad, USA) programmed with a 10 min denaturation at 95 °C, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, followed by a final extension of 10 min at 72 °C. All PCR products were checked for quality using gel electrophoresis through 1% (w/v) agarose Tris-Borate-EDTA gels stained with ethidium bromide and UV illumination. Approximate amplicon size (ranging from 300 to 1400 bp) was visualised by comparison with a DNA size standard (Hyperladder II; 50-2000 bp; Bioline, UK). PCR amplicons from the duplicate reactions were pooled and purified using a MoBio SpinClean PCR column purification (MoBio Laboratories, Inc., Carlsbad, CA, US). The concentration of amplicons were determined using a NanoDrop™ 8000 (Thermo Fisher Scientific Inc., Wilmington, DE, USA).

A volume of 2 µl of the purified DNA was mixed with 8 µl of separation gel (Fragment Analyser Qualitative Kit DNF-910-k0500, Analytical Technologies, Ames, IA, US) and separated by capillary electrophoresis (6kV for 45 minutes) in duplicate using a Fragment Analyzer™ (Advanced Analytical Technologies, Inc., Ames, IA, US) following the manufacturer's protocols. Electropherograms were analysed using PROSize (Advanced Analytical Technologies, Inc., Ames, IA, US).

Based on a separation accuracy test using DNA ladders supplied in the kit, fragment binning size of 10 bp and 15 bp were used for fragments under and above 900 bp respectively. "Shoulder peaks" which are generally 1.5 bp from a larger peak were not scored. Only fragments detected in both duplicate reactions were scored. The

cutoff criterion for peaks from baseline noise was determined at 0.9 % of total fluorescence (Luna et al., 2006). This value corresponds to the maximum number of different peaks that can be discriminated by means of the ARISA method with the primer set and the separation capacity of Fragment Analyser Qualitative Kit DNF-910-k0500.

3.3.4 16S rRNA gene pyrosequencing of larval mortality samples

Specific culture water and larval samples from cultures exhibiting high and low mortality were selected for community analysis using 16S rRNA gene pyrosequencing. The following time points were selected: 1) one day prior to onset of larval mortality (replicates H1-3 and H3-4); 2) during larval mortality (replicate H1-4 and H3-5), and 3) standard fed cultures with healthy larvae (replicate S4-4 and S6-5).

The sequence region V3 to V5 of the 16S rRNA gene was PCR amplified in duplicate reaction run using primers 341F (5-3’ direction, 5'-CCTACGGGAGGCAGCAG, Muyzer et al., 1993) and 907R (5-3’ direction, CCGTCAATTCMTTTRAGTT, Lane et al., 1985). A blocking primer (907R_block, 5-3’ direction, TGAGTTTCACCCTTGCGAGCG_C3 spacer was added at 20 times the final concentration of the amplification primers to minimise amplification of mitochondrial and chloroplast 16S rDNA from the high concentration of microalgal cells present in samples (Powell et al., 2012). PCR were carried out in 25 µl reaction volumes using 1 x Red Immomix, 0.4 µM 341F, 0.4 µM 907R, 8.0 µM 907R_block, 30-50 ng DNA template. PCRs were performed in a C1000 Touch^{TM} Thermal Cycler (Bio-Rad, USA) programmed to run 10 min denaturation at 95 °C, followed by 35

cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. Reactions were completed with a final extension of 10 min at 72 °C. Target PCRs amplicons of approximately 560 bp were checked by electrophoresis, and duplicate reactions were pooled and purified using a MoBio SpinClean PCR purification kit.

Pyrosequencing of the V3 and V5 regions covered by 341F and 907R primers (Soergel et al., 2012)was carried out by Research and Testing Laboratories (Lubbock, Texas, USA) using a Roche 454 FLX instrument with Titanium kit reagents with tagged the primers identical to those used for the 16S rRNA gene amplifications. FASTQ files were trimmed to remove primer, barcode and adapter regions using an internally developed algorithm at Research and Testing Labs. All sequences were organized by read length and de-replicated using USearch (Edgar, 2010). Clusters of sequence were developed based on seed sequences which shared ≥97 % sequence similarity. The seed sequence, or known as Operational Taxonomic Units (OTUs) was then sorted by abundance and then clustered again with a 1 % divergence cut-off to create consensus sequences for each cluster. Clusters containing only one sequence or sequences <250 bp in length were removed. Any reads that failed to be clustered (typically poor quality reads present in low numbers) were removed. Chimeric sequences were detected and removed using UCHIME in the *de novo* mode (Edgar et al., 2011). Each seed sequence was then queried against a database of high quality sequences derived from the NCBI database using a distributed .NET algorithm that utilized BLASTN+ (KrakenBLAST, www.krakenblast.com). High scores matches were grouped in terms of taxonomic hierarchy based on per cent similarity values. On very few occasions, sequences that yielded high score matches of <75 % similarity were discarded. The 16S rRNA

sequence read abundance was adjusted by dividing the sequence reads by the median number of 16S rRNA genomic copies found in each available taxon (Kembel et al., 2012, Appendix 6) based on data from the publically accessible database rrnDB at https://rrndb.umms.med.umich.edu/ (Stoddard et al., 2015).

3.3.5 Statistical analysis

Automated Ribosomal Intergenic Spacer Analysis (ARISA) data from all seawater (n=36) and larvae samples (n=36) were combined into a single fragment size and concentration matrix (nmole l^{-1}) and imported into the software package Primer-6 PERMANOVA+ package (version 6.1.12 and version 1.0.2; Primer-E, Ivybridge, UK). The data were forth-root transformed and a sample similarity matrix calculated using Bray-Curtis dissimilarity coefficiencts. The null hypothesis (no difference between samples groups i.e feeding level, sample type) was tested with default settings and unrestricted 9,999 permutation using permutational multivariate analysis of variance (PERMANOVA). Principal Coordinates (PCO) analysis was used to examine patterns of bacterial community data. A 2-dimensional canonical analysis of principal coordinates (CAP) was used to better illustrate the patterns of bacterial community shifts associated with mortality events. The seawater and larvae samples were categorised as healthy (n=60), a day before (n=4), during onset of mass mortality (n=4) and a day after mortality (n=4).

3.4 Results

3.4.1 Effect of overfeeding on larval culture bacterial community and larval mortality level

In comparison with standard fed larval cultures, overfeeding produced significantly different bacterial communities associated with both seawater (F=3.70, df 1, 34, p=0.001) and larvae (F=2034, df 1, 34, p=0.011). Overfed cultures H1 and H3 suffered cumulative mortality of over 60 % at the end of rearing (8 dpf) and both presented notable spikes in daily mortality of 31 % and 36 % in just a 24 h period at 4 and 5 dpf, respectively (see Figure 3-1). Daily mortality then continued at a lower rate (up to 17 % per d) till the end of rearing at 8 dpf. Overfed culture H2 showed lower cumulative mortality (22 % at 8 dpf) and moderate daily mortality which did not exceed 10 %. Cumulative mortality of standard fed cultures was less than 15 % at the end of rearing.

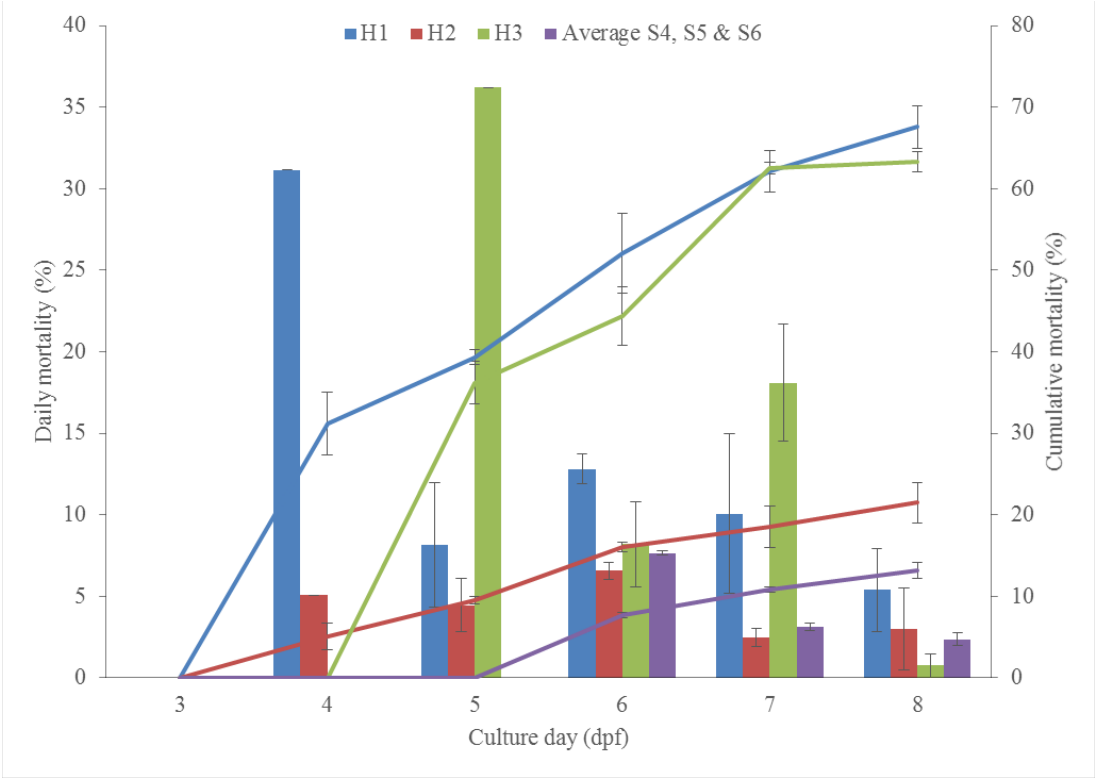


Figure 3-1 Percentage of daily (represented as histogram) and cumulative mortality (line graph) of mussel larval cultures of overfed H1 (blue coded), H2 (red) and H3 (green), and averaged of 3 standard cultures (S4, 5 and 6) reared from 3 to 8 dpf with water changes every 2 d at 4, 6 and 8 dpf. Standard error bar for the individual replicate of overfed cultures indicates mortality count variation whereas standard error bar for the averaged standard fed cultures indicates biological variation.

Overfeeding resulted in notable changes in ARISA fingerprints in comparison with standard fed cultures. Genotypes 420 and 600 were detected more frequently in seawater and were more abundant in overfed cultures (Figure 3-2a). Genotype 850 bp was detected at increasing abundance during later stages of feeding in seawater samples. The pattern of abundance of 3 genotypes- 750, 950 and 1400 were associated with mortality. These genotypes were minimal in seawater of all standard fed cultures, higher in overfed culture H2, and highest in cultures H1 and H3, which

had the highest mortalities. Similarly, genotype 1100 bp abundance peaked during highest daily mortality at 4 and 5 dpf respectively and declined in abundance after mortality at 6 dpf and onwards. However, its abundance was also high in all standard fed cultures.

In terms of larvae samples, genotypes 440, 750 and 1400 were detected more frequently and at higher concentrations in overfed culture (Figure 3-2b). Genotype 580 was absent in H2 and appeared briefly at low concentration in S3. However, it and genotype 1100 was present in H1 and H3 at higher concentrations during and after mortality. Interestingly, genotype 600 was persistent in both overfed and standard fed larvae but was at higher concentration in all standard fed cultures. Similarly, genotype 630 was detected exclusively in the standard fed cultures only.

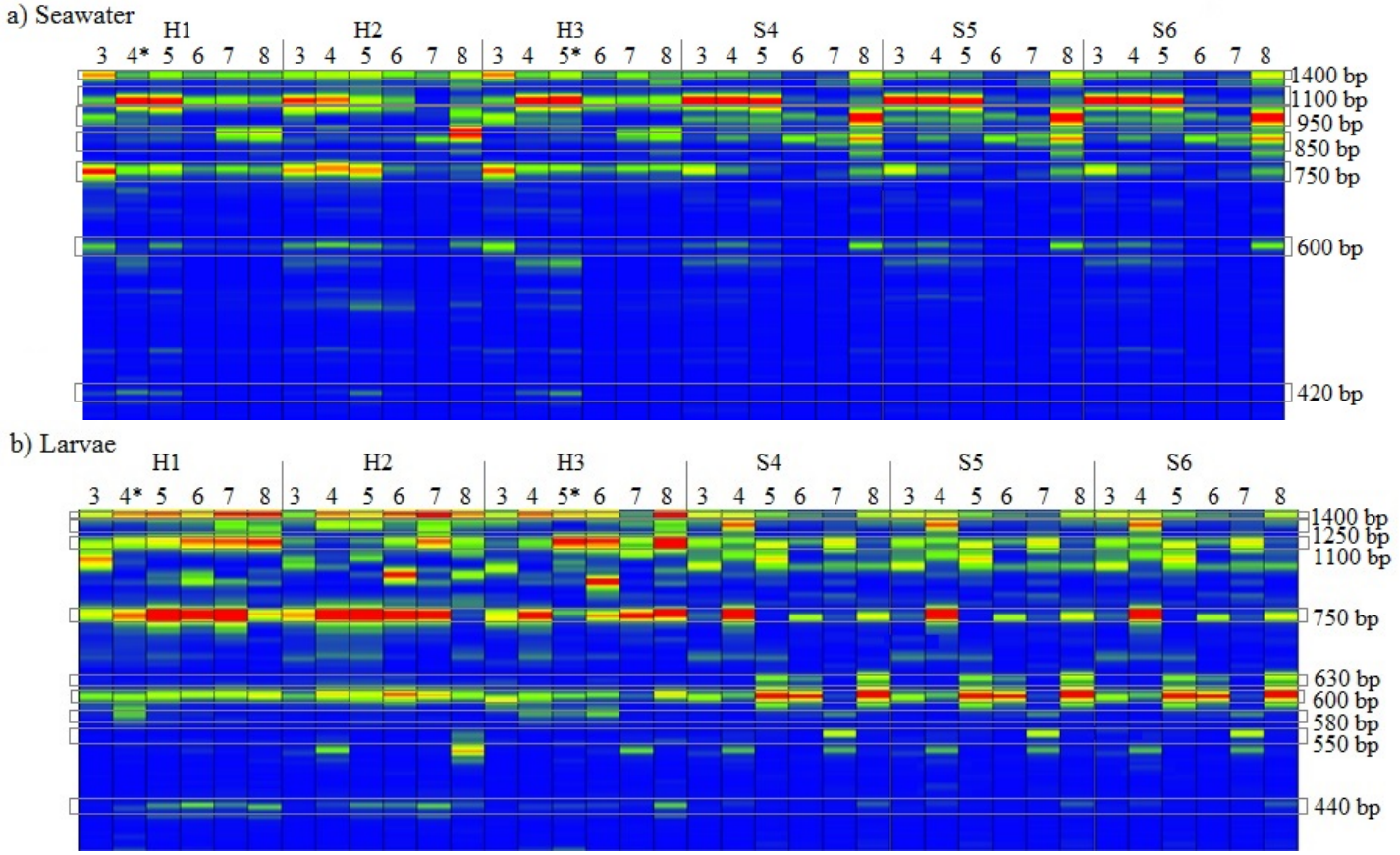


Figure 3-2 Heat map of bacterial community ARISA fingerprints of a) seawater and b) mussel larvae samples based on ARISA of 16-23S rDNA intergenic spacer 1 (ITS1) showing genotypes ranging from approx. 400 bp to 1400 bp. Samples from overfed cultures H1 and H3 that suffered high mortality at 4 and 5 dpf (asterisk) are compared with samples from overfed H2 and standard fed S3, S4 and S6 cultures that had low mortality levels. Number on top of each lane indicates culture age as dpf.

3.4.2 Bacterial community structure changes associated with larval mortality

Principal Coordinates (PCO) analysis of all 3 over and 3 standard fed larval cultures bacterial community data (n=72) showed notable community clusters corresponding to seawater and larvae samples (see dotted circles in Figure 3-3). The samples associated with high mortality in overfed culture H1 and H3 (n=6, see coloured plots) were observed to shift towards the bottom of the plot leading up to onset of mass mortality (>30 % in a day) and returned to the centre of each community cluster a day after mortality onset. Samples collected during mortality occupied the lowest part of the plot where seawater and larval bacterial communities became more similar to each other.

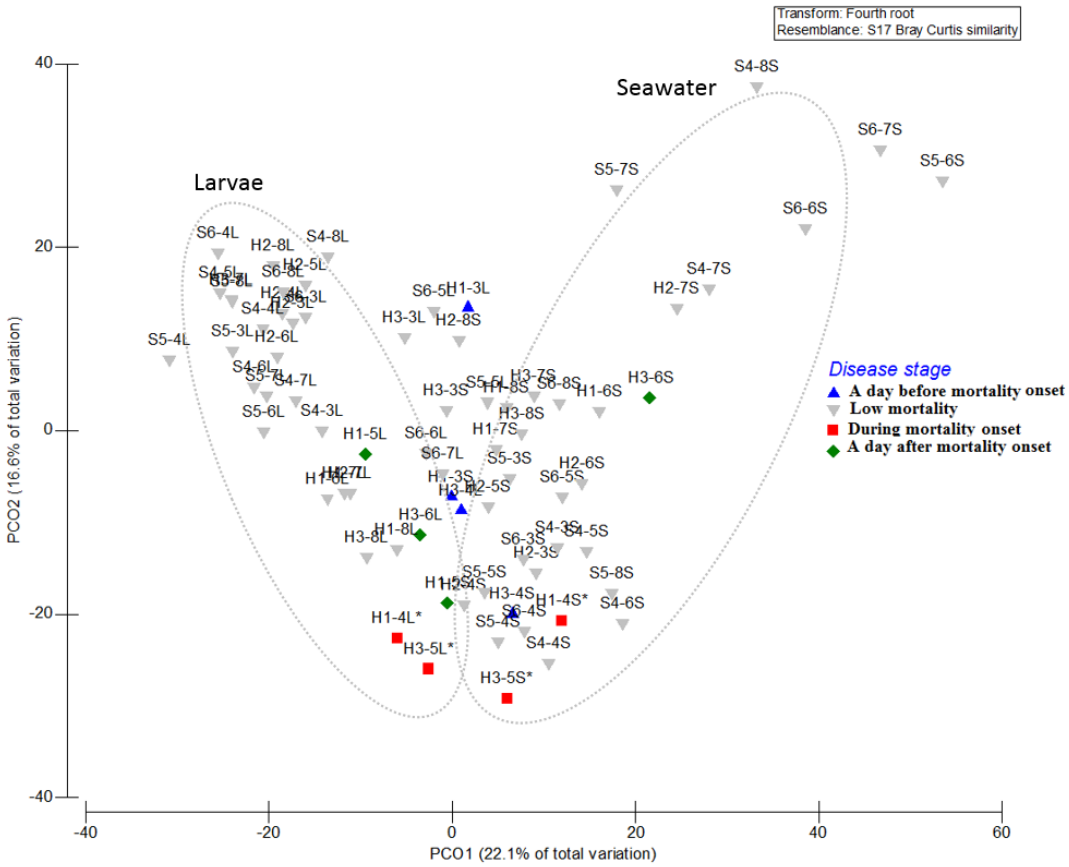
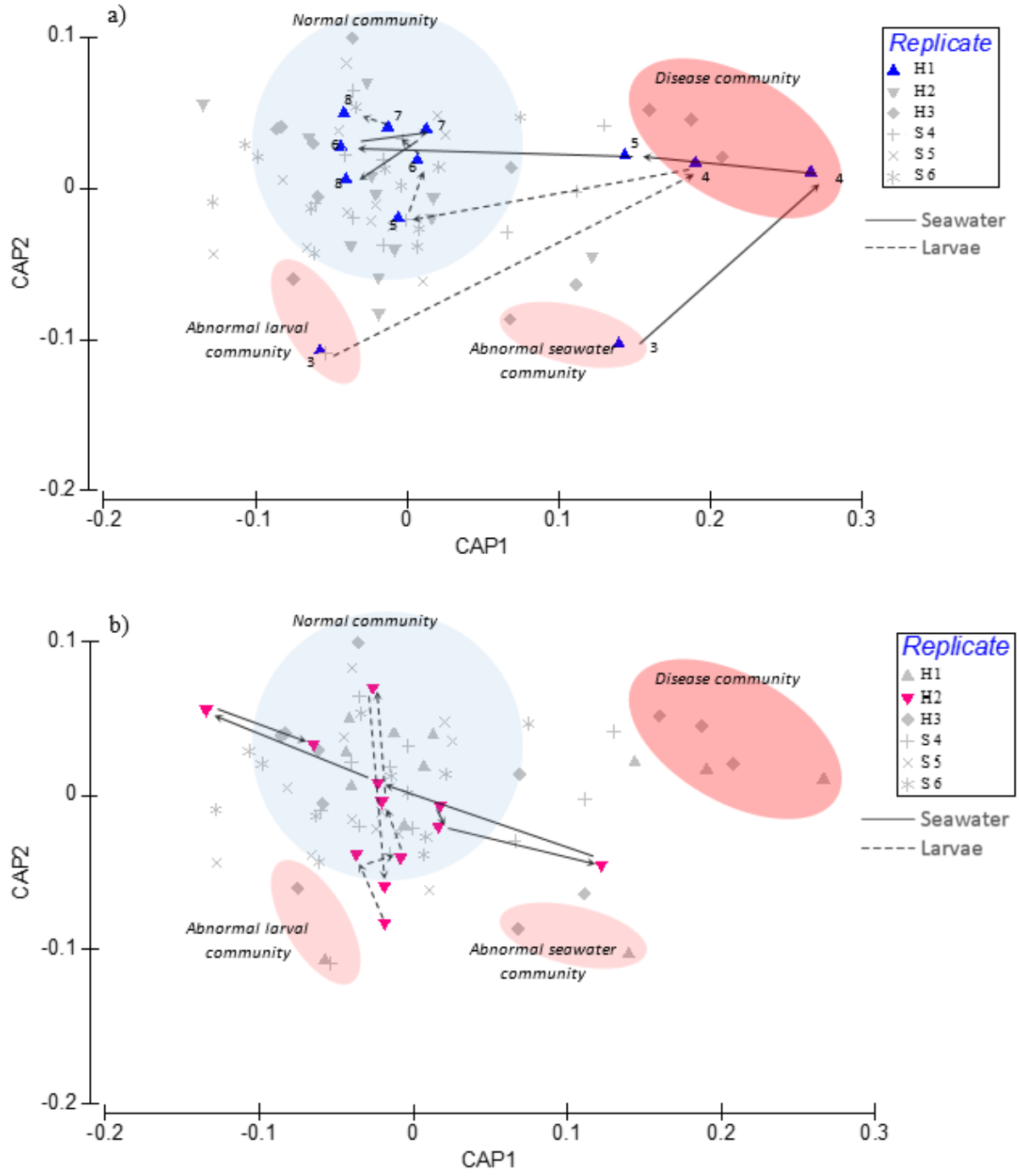


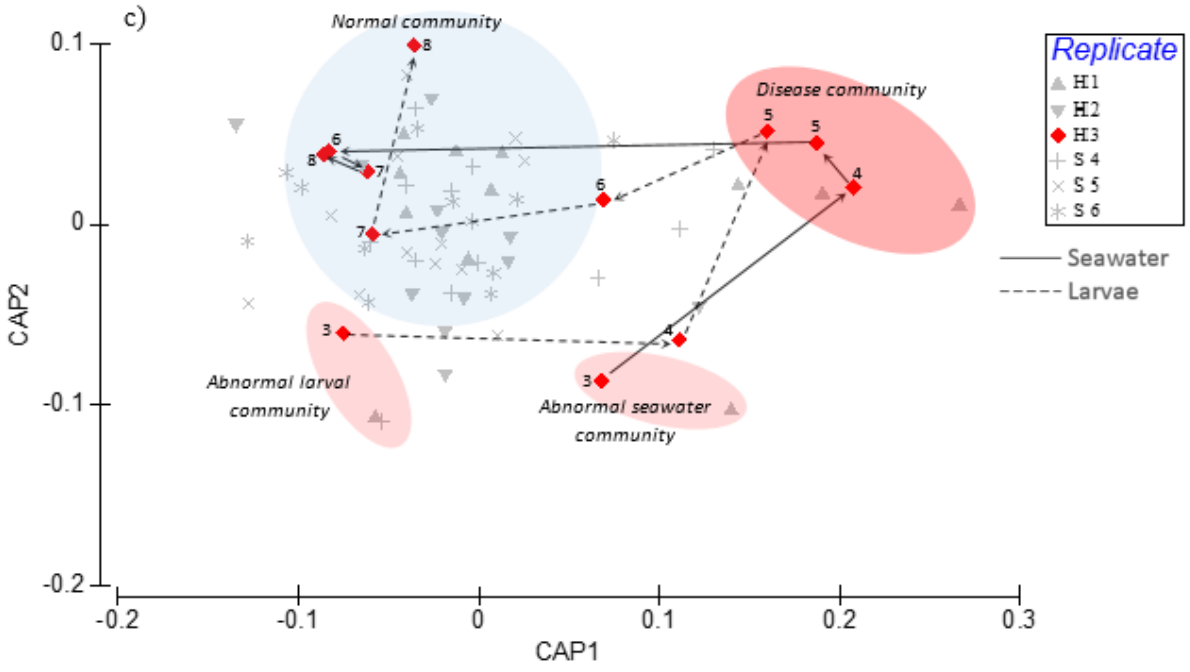
Figure 3-3. Principal Coordinates (PCO) analysis of all 3 overfed (H1, H2 and H3) and 3 standard fed mussel larval cultures (S4, S5 and S6) reared from 3 to 8 dpf. A total of 72 ARISA data samples (36 seawater, S; 38 larvae, L) were included. Plots were labelled based on culture (e.g. H1), day of rearing (e.g. 3) followed by sample typle (e.g. L). Seawater and larval associated bacterial communities whether in over and standard fed cultures were significantly different to each other (F=14.3, df 1, 70, *p*=0.0001) as reflected by clustering of plots based on seawater and larvae sample type (see dotted boundaries). Samples from cultures showing healthy/low level of mortality and, H1 and H3 associated with high mortality (>30 %) at 4 and 5 dpf are coded as follows: Grey = Low (healthy) mortality; Blue = one day prior to onset of mass mortality; Red = during high mortality; Green = one day after mortality. Principal Coordinate axis 1 and 2 explained 22.1 % and 16.6 % of total variation respectively.

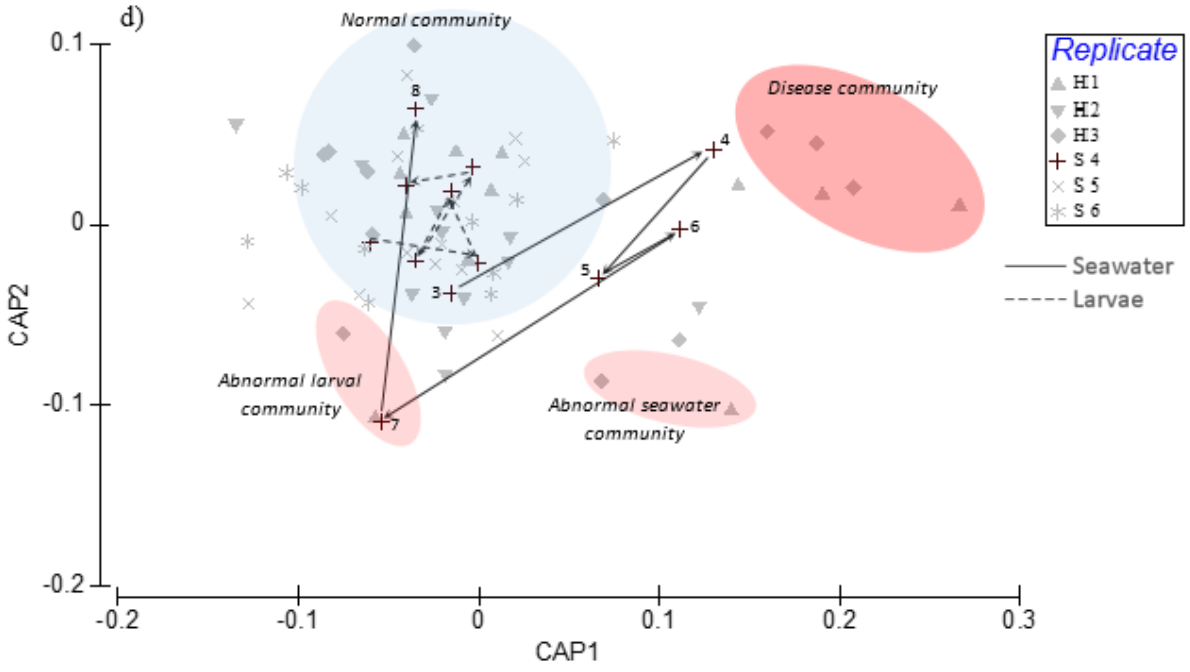
3.4.3 Bacterial community structure shifts

The clustering of mortality data were furthered examined using CAP to investigate the classification of communities against defined factors. CAP analysis examined 4 disease developmental stages: 1) healthy (n=60), 2) a day before mortality (n=4), 3) during mortality (n=4) and a day after mortality (n=4) demonstrated systematic shifts in seawater and larval bacterial communities corresponding to the course of mortality. The clustering of samples in the CAP ordination plots showed consistent shifts in communities closely synchronised with the development and onset of larval mortality in the overfed cultures (Figure 3-4). The non-mortality and mortality associated communities were clearly separated along the CAP1 axis with most non-mortality communities clustering in the centre of the plot (normal community cluster), while mortality-associated samples clustered to the left of the plot (disease cluster) (Figure 3-4). In cultures H1 and H3, seawater communities were displaced toward the bottom of the plot compared to other cultures at 3 dpf and progressed towards the “disease cluster” during 4 and 5 dpf (see solid-line trajectories in Figure 3-4a and 4c). The larval communities then followed similar progression observed in the seawater communities. At 3 dpf the communities were closer to the normal cluster (see dotted line trajectory in Fig.4a and 4c) and required up to 2 d to reach the disease cluster (see dotted line Figure 3-4c).


a)
Normal community
Disease community
Abnormal larval community
Abnormal seawater community
Replicate
H1
H2
H3
S4
S5
S6
Seawater
Larvae
CAP2
CAP1
0.1
0
-0.1
-0.2
-0.2
-0.1
0
0.1
0.2
0.3
b)
Normal community
Disease community
Abnormal larval community
Abnormal seawater community
Replicate
H1
H2
H3
S4
S5
S6
Seawater
Larvae
CAP2
CAP1

(continued)


c)
Normal community
Disease community
Abnormal larval community
Abnormal seawater community
Replicate
H1
H2
H3
S 4
S 5
S 6
Seawater
Larvae
CAP2
CAP1
0.1
0
-0.1
-0.2
-0.2
-0.1
0
0.1
0.2
0.3


d)
Normal community
Disease community
Abnormal larval community
Abnormal seawater community
Replicate
H1
H2
H3
S 4
S 5
S 6
Seawater
Larvae
CAP2
CAP1
0.1
0
-0.1
-0.2
-0.2
-0.1
0
0.1
0.2
0.3

(continued)

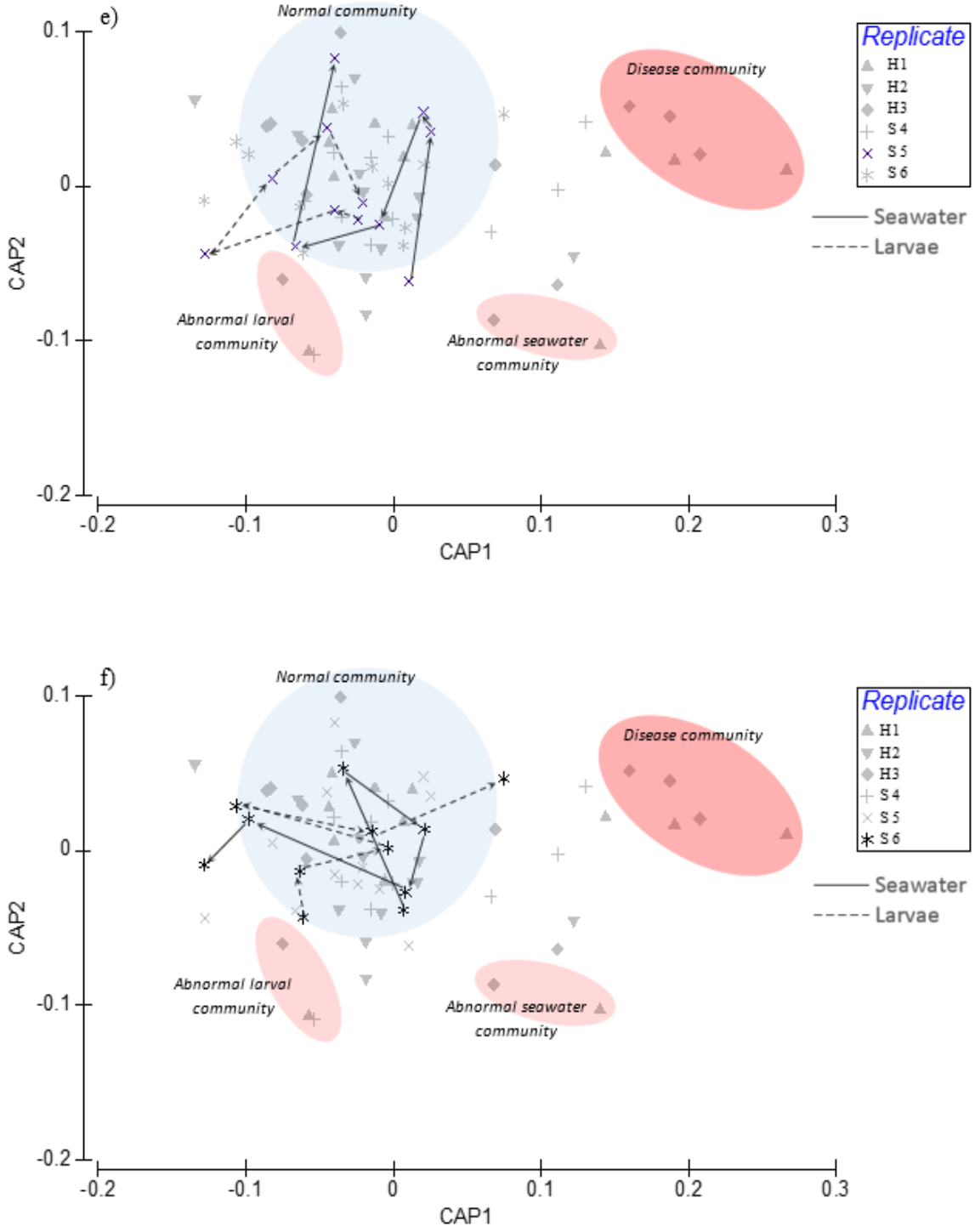


Figure 3-4 Six panels of canonical analysis of principal coordinates (CAP) each showing the complete ARISA fingerprint data (n=72) generated based on development of disease stage classified as normal, a day before, during and a day after mass mortality. Data plot for each culture is highlighted for overfed culture a) H1, b) H2, c) H3, and standard fed culture d) S4,

e) S5 and f) S6 against a background of greyed out data plots. Solid and dotted line trajectory tracks shifts of bacterial community for seawater and larvae respectively starting from 3 to 8 dpf and are numbered in panel a), c) and d). Data plots are overlaid with boundaries to represent normal community (seawater and larvae inclusive) which dominated the plots, abnormal H1 and H3 larval and seawater community, and diseased community where mass mortality events were observed in H1 and H3 at 4 and 5 dpf.

Notably in the disease cluster comprising communities associated with rapid mortalities, seawater and larvae samples communities of H1-4 and H3-5 were more similar to each other, as reflected in the earlier PCO analysis (Figure 3-3). Examination of community similarity of paired seawater-larval sample based on ARISA dataset (n=36 pairs) revealed mortality samples H1-4 and H3-5 possessed highest Bray Curtis similarity coefficients at 68 % and 81 % whereas healthy samples averaged 45±2 % (Figure 3-5).

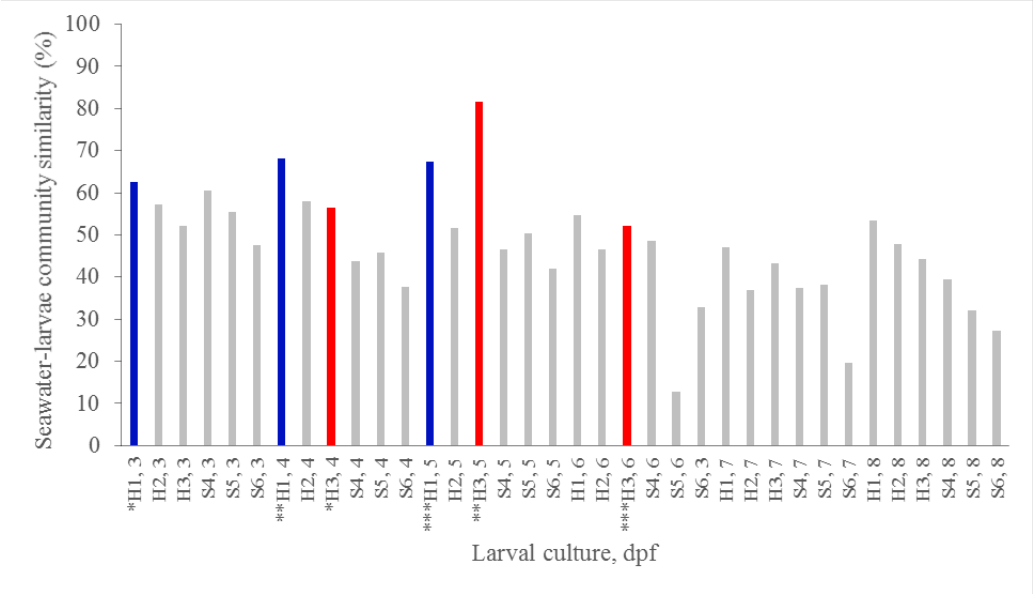


Figure 3-5 Pairwise similarity seawater and larvae bacterial community based on Bray Curtis matrix of ARISA data (n=72) of overfed culture H1, 2 and 3, standard fed culture S4, 5 and 7 reared from 3 to 8 dpf. Mortality samples are coded blue for H1, and red for H3. * indicates pairwise similarity at one day before mass mortality, ** during mortality and * a day after mortality.**

Based on the CAP analysis, cultures H1 and H3 which suffered the severest mortality levels developed abnormal seawater larval communities at about 3 dpf, and that mortality only occurred when both seawater and larval communities converged to a specific disease state. Culture H2, which did not suffer elevated mortality (i.e daily mortality no more than 10 %), did not show abnormal communities as seen in H1 and H3, despite the H2 seawater sample being briefly near the disease cluster at 5 dpf (Figure 3-4b). It is, however, interesting that the seawater community of S4 unlike S5 and S6 deviated towards the disease cluster at the start of rearing and spent up to 4 d in between the healthy and disease cluster before reverting back to the healthy community cluster at 8 dpf. Culture S4 did not show any elevated mortality

level.

Reduced daily mortality in cultures H1 and H3 from 5 and 6 dpf onwards were clearly characterised by the return of seawater and larval communities from the right hand side cluster (disease state) back to the central cluster (healthy state), and remained that way until the end of rearing.

3.4.4 Bacterial composition comparison and shifts

In terms of bacterial diversity at class level (Figure 3-6), 16S rRNA gene tag 454 pyrosequencing of samples from mortality cultures H1 and H3 (a day before and during), and healthy cultures S4 and S6 showed comparable dominance of sequence reads belonged to class *Alphaproteobacteria* (range of 55 to 69 % of total sequence reads) and *Gammaproteoobacteria* (0.5 to 2.2 %). However, bacterial composition were more variable at genera level. In the *Alphaproteobacteria* (Figure 3-7a), reads affiliated with *Sphingomonas* was relatively more abundant at an average of 21.6 % and 20.3 % in the seawater and larval samples from the healthy cultures compared with those from a day before (0.1 %) and during mortality cultures (7.7 %). Similarly, reads affiliated with *Methylobacterium* was also relatively more abundant in the healthy cultures at 2 % and 5 % in the seawater and larval samples respectively. In contrast, reads affiliated with *Thalassobius* and *Phaeobacter* were consistently more dominant in both seawater (33 %) and larval (15 %) samples from cultures during mortality.

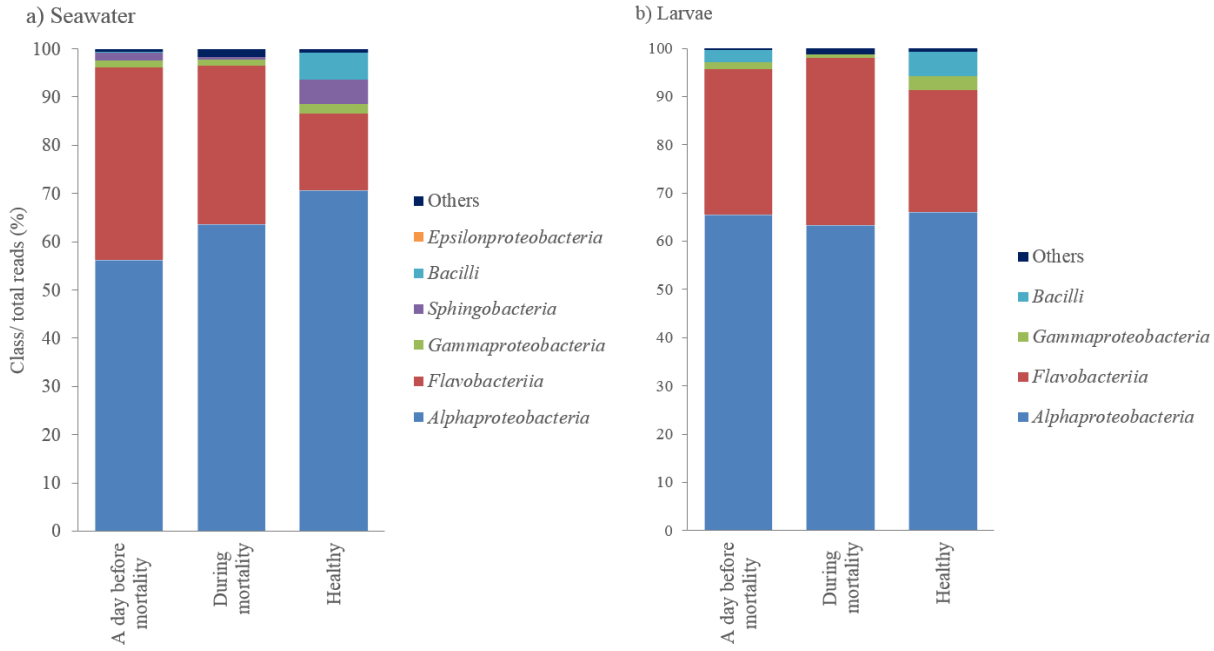


Figure 3-6 Bar graph showing average percentage of composition of bacterial communities in a) seawater and b) larval associated samples based on aggregation of amplicon pyrosequencing reads organised to class level. The 'Others' contains *Cytopagia, Bacteroidia, Deltaproteobacteria, Betaproteobacteria, Actinobacteria, Clostridia and Phycisphaerae*.

In the *Gammaproteobacteria* (Figure 3-7c), *Alteromonas* and *Pseudoalteromonas* affiliated reads appeared to be relatively more abundant in the healthy larval samples (both accounted for 2.3 %) compared with less than 0.25 % from the mortality cultures (a day before and during mortality). *Neptuniibacter* affiliated reads were a dominant proportion of the *Gammaproteobacteria* in the seawater communities regardless of the health status. However, it appeared to be only detected in the larval samples from cultures a day before and during mortality. Similarly, reads affiliated with *Vibrio* were below detection in the healthy samples but were detected at range 0.05 % to 0.25 % in the larval and seawater samples.

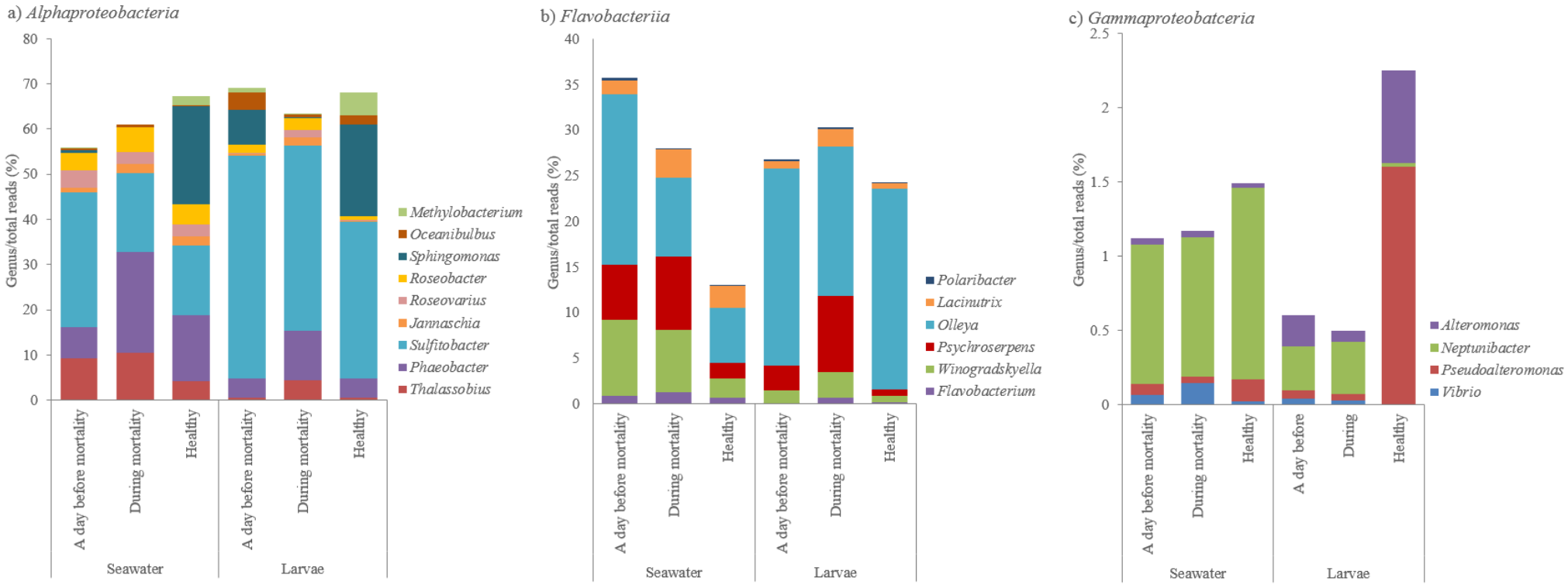


Figure 3-7 Bacterial diversity assemblages (%) at the genus level for a) *Alphaproteobacteria* b) *Flavobacteriia* and c) *Gammaproteobacteria* in seawater and larvae samples associated with a day before mortality, during mortality and healthy cultures. The focus of the 3 bacterial classes is because they account for > 95 % of the total sequence reads.

It is observed *Flavobacteriia* affiliated reads were relatively more abundant in the seawater from mortality cultures compared with those from healthy cultures (over 30 %versus 15 % , **Error! Reference source not found.**a). Further examination (Figure 3-7b) showed the increase in *Olleya* in mortality associated seawater samples (over 15 %) was difficult to correlate to mortality as it was comparably abundant (over 20 %) in the healthy samples. Notably, *Psychroserpens mesophilus* increased in relative abundance with development of mortality and were consistently reflected in both the seawater and larvae (**Error! Reference source not found.**b). Examination of individual mortality cultures (**Error! Reference source not found.**) showed *Psychroserpens* in the seawater samples increased from 6.3 % and 6.5 % in culture H1 and H3 at a day before mortality to 9.1 and 8.9 % during mortality respectively. Similarly, larval-associated *Psychroserpens* increased from 0.3 % and 6.4 % in H1 and H3 at a day before mortality to 8.4 % and 9.3 % at times of mortality. These represented a notable shift in relative abundance when compared with culture S4 and S6 larvae (2.5 % and below detection respectively).

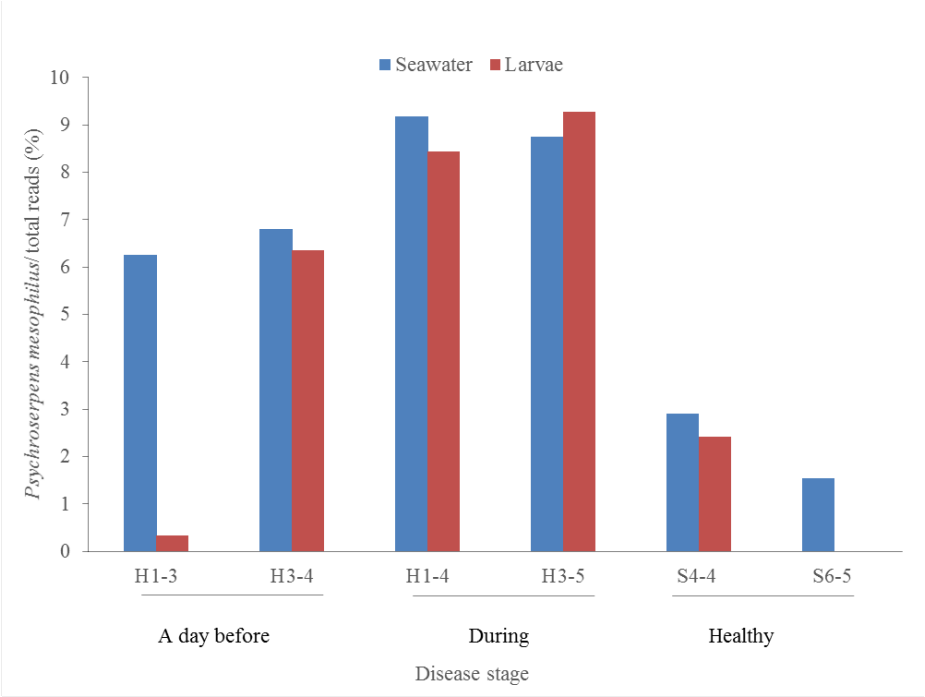


Figure 3-8 Percentage of *Psychroserpens mesophilus* affiliate reads of total species reads based on 16S rRNA gene pyrosequencing in seawater and larvae sample of overfed cultures H1 and H3 a day before onset of mortality at 3 and 4 dpf; during mortality at 4 and 5 dpf compared with optimally fed and healthy cultures S4 and S6 at 4 and 5 dpf, respectively.

3.5 Discussion

This study attempted to determine if and how mortality in early stage mussel larval cultures is associated with changes in microbial communities. This study observed overfeeding unpredictably resulted in mass mortality (> 60 % at the end of rearing) in 2 out 3 overfed culture cultures. The microbial data consisted of 2 high mortality and 4 healthy cultures were analysed using unconstrained ordination procedures and the patterns of clustering of bacterial community associated with mortality events were compared using parametric Principal Coordinates (PCO) and non-parametric Principal Component Analysis (PCA). As a pattern of community segregation is

reproducible in the 2 ordination techniques (see Figure 3-3 and Appendix 4) and the timing of abnormal community shifts as well as mortality events coincided with the growth spikes of culturable *Vibrio* in Chapter 2, CAP was used as additional aid to illustrate the pattern of bacterial shifts.

In this study, over feeding mussel larval cultures with microalgae increased the risk of mortality because in contrast, all standard fed cultures S4, S5 and S6 and overfed culture H2 had good survival and produced healthy larvae at the end of rearing at 8 dpf. Whilst this study did not monitor water quality parameters, larval death is unlikely caused by direct effect of overloading of algal cells because overfed culture H2 showed good survival. Deterioration of physical water quality to the extent of causing mortality is unlikely as Eggermont et al. (2014) demonstrated addition of peptone as organic matter to stimulate bacterial cell multiplication did not adversely affect dissolved oxygen (DO), pH, nitrite and total ammoniacal nitrogen.

The two events of mortality in larval cultures H1 and H3 resemble bacillary necrosis based on its high mortality rates (Nicolas et al., 1996; Kesarcodi-Watson et al., 2009b) and the fact they were closely linked to bacteria. This study demonstrated for the first time shifts in seawater and larval bacterial communities leading up to larval mortality followed by restoration to healthy communities as mortality subsided. Larval cultures suffering a high mortality developed abnormal seawater and larval communities as early as 1 d after being overfed before poor larval survival was evident. This suggests that despite the seemingly sporadic and rapid mortality commonly reported (for example Chapman, 2012), bacillary necrosis undergoes a developmental period which is not always detected using culture-based techniques

(Disalvo et al., 1978; Nicolas et al., 1996; Sugumar et al., 1998b; Sung et al., 2001; Chapman, 2012). It is suspected that bacterial factors contributing to the abnormal communities may have played a greater role over overfeeding in the occurrence of bacillary necrosis in this case because larval culture H2 despite being a replicate to overfed cultures H1 and H3 did not have the abnormal communities seen in H1 and H3 (Figure 3-4b) and showed good survival. The cause or source of community variation in H2 is not known given that they were essentially biological replicates, but this reinforces the importance of stochastic bacteria/community factors in interacting with overfeeding to cause disease.

Interestingly, our study as seen in H3 particularly showed that the abnormal communities associated with seawater and larvae were surprisingly persistent despite total seawater changes. The water changes in H3 at 4 dpf (Figure 3-4c) had no clear effect as seawater communities at 5 dpf as they were adjacent to 4 dpf communities while the larval communities on the next day at 5 dpf, shifted into the disease cluster. The explanation to the persistent communities is possibly to do with selective pressures associated with H1 and H3 culture environment and not the feed input and replacement seawater as all experimental larval culture units used the same larval and seawater source. It is possible the bacterial communities transfer from the larvae, biofilms on the surface of the culture containers, or a combination of both. The suspicion of the role of biofilm was also raised by Sandaa et al. (2003) where the authors observed water communities at the beginning of intake pipe changed significantly at the end of the delivery line in a scallop hatchery. The container surfaces and larvae were not rinsed/disinfected during water changes, therefore the microbial community established on the surface of the containers were not

removed. As a result, the attached community was likely to be have been carried over between water changes and perhaps rapidly re-colonise the seawater and ensure continuity to a disease community.

Standard fed culture S4 actually had the first rearing day (3 dpf) of normal seawater community as starting inoculum, but rapidly deviated to an abnormal community in the next 24 h (see solid trajectory lines at 3 and 4 dpf of Figure 3-4c). However, the seawater associated communities after water changes at 4 and 6 dpf reverted the communities shifts closer to the normal/healthy community cluster (see solid trajectory lines at 4, 5, 6, and 7 dpf).

The success of water changes in this case as opposed to H1 and H3 were interesting and suggests the selective pressure sustaining bacillary necrosis was weaker in S4 compared with H1 and H3, indicating that water changes is effective against early stage of disease where only the seawater is infected. The same could have been observed in H1 and H3 if water changes were made every day instead of every 2 d if the disease were meant to be prevented. Nevertheless, our daily examination of larvae did not provide any indication of early disease development.

Infection sources of bacillary necrosis are commonly suspected or reported to be algal feed, broodstock, larvae and seawater (Prado et al., 2005; Eggermont et al., 2014; Dubert et al., 2015). In this case, it is difficult to ascertain the source of causative agents because bacterial communities associated with seawater ,larvae and algae prior to start of larvae were not examined. However, seawater as sources/medium of infection is possible even though it received 1 µm filtration and UV irradiation which is considered to be of industry standard and routinely used in

commercial bivalve hatcheries (Powell et al., 2013). This bacterial community analysis showed that at 3 dpf, abnormal seawater communities H1 and H3 were closer to the disease communities than that of larvae indicating that seawater were quicker in becoming abnormal before then infecting the larvae. The infection route was demonstrated when culture H3 larvae progressed from abnormal larval communities at 3 dpf to becoming very similar to abnormal seawater community at 4 dpf before mortality occurs at 5 dpf. The same could have happened in H1 but the transition to the disease community was completed in just 2 days. The consistent pattern of seawater community changes prior to mortality may suggest that larvae are colonised by seawater community such as by strains related to the *Psychroserpens mesophilus* (**Error! Reference source not found.**), prior to and during the mortality events. This could be interpreted as an invasion of bacteria and/or changes in seawater bacterial activities (such as toxic bacterial metabolites production) that compromises immunity of larvae that are unable to resist colonisation by the seawater community.

This study shows alterations in seawater bacterial communities is correlated with poor larval health and this agrees with several studies (Preheim et al., 2011; Wendling et al., 2014). Such observations pose renewed concern of larval mortality associated with seawater bacteria in hatcheries. The former authors observed that various body compartments and even the digestive system of adult blue mussels surprisingly showed limited host preferences for diversity of bacteria. They similarly demonstrated *Vibrio* communities in the hemolymph of Pacific oysters is not stable and depends on *Vibrio* spp. taken up from the surrounding water. These indicate the bacterial communities of bivalve are assembled from ambient seawater

communities and therefore closely reflect changes in seawater environment, potentially highlighting seawater-to-mussel larval interactions.

An important characteristic of bacillary necrosis is its sporadic nature. In this experiment the rate of disease development and ultimately timing of mortality was reflected by the rate of change of the larvae microbial community. Such correlation is supported as mortality only occurred when both the larval and seawater communities converged on the disease community state. Despite a similar starting state, high mortality in H3 was delayed compared with H1 and was correlated with a similar delay in change of the larval associated community to a microbial community similar to seawater (see Figure 3-4c). It is uncertain, however, whether larval death is caused by a specific pathogen or overall microbial shifts that are detrimental.

The lessening of mortality rates in H1 at 5 and H3 at 6 dpf was reflected in the restoration of both seawater and larval communities to the initial normal state. The recovery is likely due to dissipation of selective pressure for disease and not by water changes as communities post onset of mortality were rapidly restored before water changes at 6 dpf (Fig. 4a and c). This could be due to larval culture microbial communities being constantly maintained by interactions of basal ecological pressures, which overcome weakened disease pressures. This study suspects that under normal larval rearing circumstances, these ecological pressures would produce seawater environment suitable for larvae, but overfeeding as shown here presents temporary but sufficient disturbance to the normal conditions causing bacterial community alterations that are potentially unfavourable to larvae.

Based on interpretation of the data, a diagrammatic representation of infection processes of bacillary necrosis in mussel larval rearing is presented (Figure 3-9). None of the aspects of these observations has ever been reported elsewhere and preliminary re-examination of data from a similar studies in an oyster hatchery by Powell et al. (2013) did not show similar observations. Therefore more research is necessary to advance these findings, but the convergence of communities observed so far could be an important microbial signature of bacterial mussel larval disease. The convergence of communities during mortalities is possible and could be interpreted as outgrowth of certain bacteria in larval cultures overgrowing the preceding seawater and larval communities.

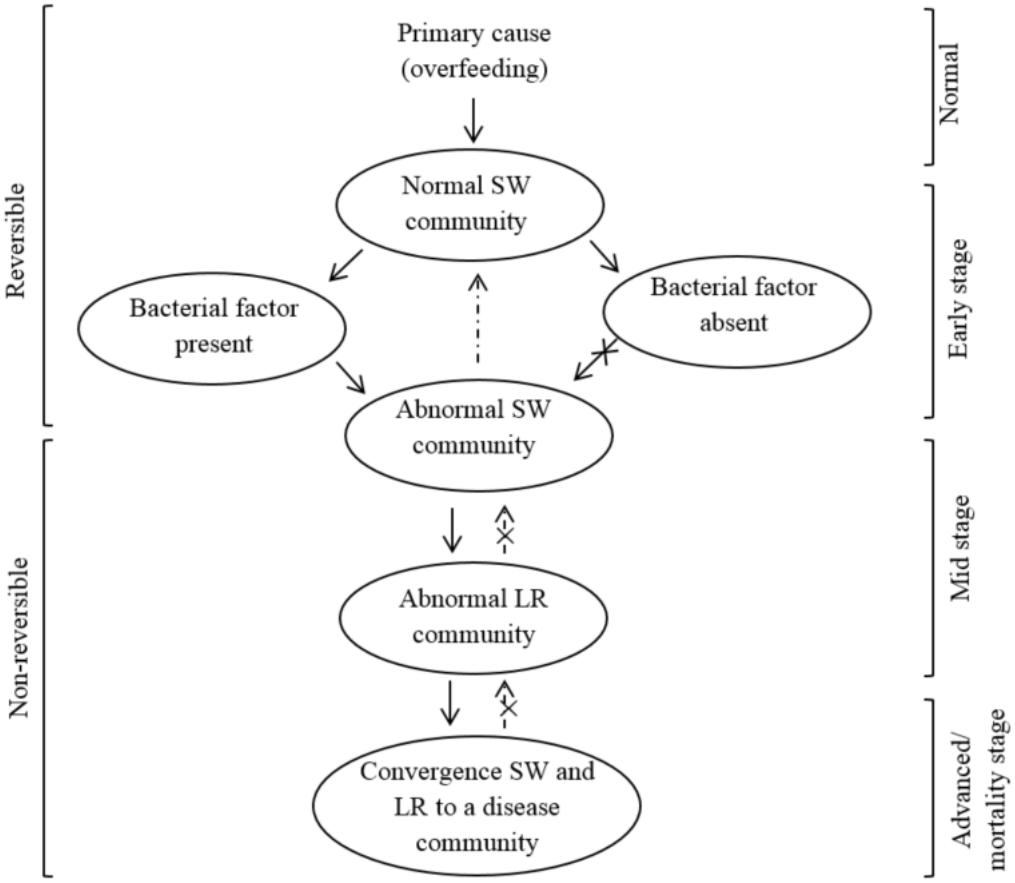


Figure 3-9 Manner of development and stages of bacillary necrosis in the first week post fertilisation larval rearing of Australian blue mussel (*Mytilus galloprovincialis*) using static cultures. Overfeeding as the primary cause alters the seawater (SW) associated bacterial community in the presence of appropriate bacterial factor (such as specific diversity assemblages) to produce abnormal seawater communities. This represents early disease stage which can further infect larval (LR) community unless prevented by regular water changes (dotted arrow). The infection of larvae considered as the mid stage of disease is non-reversible by water changes (crossed dotted line). The rate of infection of larvae would determine the timing of larval mortality which is characterised by the convergence of seawater and larval bacterial communities to a specific disease community. The post mortality community is restored to the initial healthy state presumably by natural equilibrium.

Examination of the 16S rRNA gene sequence reads shows the convergence of seawater and larvae to a specific diseased bacterial community at the onsets of mortality (Figure 3-4a and c, and Figure 3-5) coincided apparently with the highest relative abundances of *P. mesophilus* affiliated reads, in both the seawater and larval communities. This preliminary association of *Psychroserpens mesophilus* indicates value to further investigate its population dynamic and potential links with bacillary necrosis.

It is worthy of mention that members of the genus *Psychroserpens* so far have not been associated with diseases in marine bivalves and this is therefore the first report. However, phylotypes of *Psychroserpens* dominated microbial communities of Atlantic salmon of naturally and laboratory infected with Amoebic Gill Disease (AGD) (Bowman and Nowak, 2004). Gills of all diseased fish were dominated (ranged from 19 to 92%) by two phylotypes of *Psychroserpens* sp. and consistently absent in healthy fish. The fact that animals in both studies were reared in DOM rich seawater due to high stock density and feeding, and the association of *Psychroserpens* in diseased animals point to the importance of nutrients in altering microbial-larval interactions. The association and potential role of *Psychroserpens* in opportunistically causing or facilitating mussel larval mortality warrants additional study.

The lack of sequence reads affiliated with *Vibrio* in the mussel larval cultures, even at the times of mortality (less than 0.25 % and 0.05 % in the seawater and larvae respectively) suggests proliferation of *Vibrio* (over 30 % Vibrio/TVC) as detected using plate counts in Chapter 2 may in fact represents a small subset of the wider

changes in the total bacterial communities. The under representation of *Vibrio* in the non-culturable communities during mortality in mussel larvae is not unexpected. Several studies using different culture independent techniques show *Vibrio* at the times of bacillary necrosis comprises less than 1 % of total bacterial communities in larval cultures of oyster (Chapman, 2012; Powell et al., 2013) and scallop (Sun et al., 2016). The comparably low proportion of *Vibrio* in several reared bivalve species associated with disease may require researcher to rethink whether abundance rather than proportion of *Vibrio* has more importance although not necessarily as causative agents.

3.6 Conclusion

This study observed that bacillary necrosis can be triggered by overfeeding of microalgae during mussel larval rearing in the first week post fertilisation. The findings describe for the first time the initial development of bacillary necrosis to the climax of disease showing mass mortality, and re-equilibrium of disease back to a normal bacterial community state. Overfeeding produces changes in seawater communities followed rapidly (<24 h) by similar changes in larval associated communities and subsequent larval mortality. Water changes are able to minimise deviation of healthy seawater community and can halt changes, and progression of infection of larvae. Sequence reads affiliated with *Psychroserpens mesophilus* were associated with onset of mortality warranting further examination to confirm this preliminary associations to better understand roles and interactions with mussel larvae.

Chapter 4 Bacterial community composition and proteolytic activity associated with bacillary necrosis in blue mussel (*Mytilus galloprovincialis* Lamarck) larval culture

4.1 Abstract

Earlier chapters indicated the progression of bacillary necrosis in bivalve larval cultures follows a consistent pattern of changes in bacterial communities that is initiated in culture seawater. However, bacillary necrosis remains unpredictable in both timing and scale of larval mortality, suggesting that seawater is a potentially important source of variation. To better understand variation in onset and progression of bacillary necrosis this study examined larval mortality events and microbial community compositions of 19 replicate controlled small-scale mussel larval cultures. To minimize microbial community variation of the seawater, each culture contained 10 larvae ml^{-1} of 0.22 µm filter sterilised 35 ppt seawater in a sterile 60 ml container. Commercial microalgal paste was added to give a final algal concentration of 7.2×10^4 cells ml^{-1}. The proportion of immotile and dead larvae was determined every 24 h. At 48 h of rearing, 2 cultures exhibited moderate to high mortality (≥50 to <80 %) and 2 cultures suffered almost complete mortality (≥ 98 %). Based on plate counts, higher *Vibrio* relative abundance in seawater and larvae was more frequently observed in mortality cultures. The level of seawater *Vibrio* relative abundance however was not consistently associated with mortality level. Principal

Coordinates (PCO) plots of Illumina MiSeq derived 16S rRNA gene taxa-abundance data show cultures with elevated mortality (≥50 to <80 % and ≥98 %) were different most notably in larval associated bacterial communities. Linear discriminant analysis effect size (LEfSe) focusing on larval community data show *Marinomonas* and *Gracilibacteria* affiliated reads were statistically more abundant in high mortality larval cultures (18.5±6.8 % versus 4.1±1.8 %, and 6.5±2.7 % versus 1.0±0.8 %, respectively) whilst *Pseudoalteromonas distincta* (40.5±6.0 % versus 4±0.9 %), *Phaeobacter gallaenciensis* (2.7±0.3 % versus 0.3±0.1%) and *Vibrio splendidus* affiliated reads (2.7±0.8 % versus 0.4±0.2 %) predominated low mortality cultures. Pair-wise one-way ANOVA shows total proteolytic activity (TPA) in rearing seawater of 3 out of the 4 elevated mortality cultures were statistically higher (0.66 mg ± 0.06 trypsin eq. l^{-1} compared with the 15 low-mortality cultures (0.33 mg trypsin equiv. l^{-1} ± 0.01). Despite the use of highly controlled seawater quality, the sporadic nature of bacillary necrosis suggests onset of bacillary necrosis is influenced by subtle variation/s in the larval culture environment. Once change is initiated in the bacterial community, the community can progress rapidly to bacillary necrosis. The association of mass mortality with elevated proteolytic levels in spontaneous bacillary necrosis in larval culture is novel and warrants more works to understand its role in mass larval mortality.

4.2 Introduction

One of the most serious problems affecting bivalve larval production worldwide is stock losses due to bacterial diseases, primarily bacillary necrosis (Paillard et al.,

2004; Travers et al., 2015), a lethal bacterial disease usually connected to various species of the genus *Vibrio* (Tubiash et al., 1965; Garland et al., 1983). Bacillary necrosis affects hatchery reared bivalves of all species including oysters (*Crassostrea* spp.; Brown and Losee, 1978; Disalvo et al., 1978; Garland et al., 1983; Lodeiros et al., 1987; Prado et al., 2005), clams (*Ruditapes* spp.; Gomez-Leon et al., 2005; Antonio Guisande et al., 2008), scallop (*Pecten* spp.; Nicolas et al., 1996; Lambert et al., 1998; Jorquera et al., 2004) and mussel (*Perna* spp.;Kesarcodi-Watson et al., 2009a; Kesarcodi-Watson et al., 2009b).

Protease production is considered a major virulence factors of pathogenic bacteria associated with bacillary necrosis (Binesse et al., 2008; Saulnier et al., 2010; Labreuche et al., 2010; De Decker et al., 2013) which facilitates bacterial invasion by causing tissue damage of protein structural components of bivalve larvae (Hasegawa et al., 2008; Labreuche et al., 2010). Given the known contribution of proteases to larval mortality, proteolytic activity of larval culture associated bacterial communities may provide an integrated measure of virulence, and thus a method for early detection of necrotic disease in larvae.

Correlation of larval mortality in commercial hatcheries with estimates of total bacterial load such as total viable counts is difficult to achieve (Nicolas et al., 1996; Chapman, 2012). To date, mass mortality events are associated with multiple bacterial types and significantly different seawater associated bacterial communities in each case (such as Chapman, 2012; Powell et al., 2013), indicating that bacillary necrosis is not caused by a particular species but can arise from within microbial

assemblages of larval cultures (Powell et al., 2013).

Even with seawater treatment systems, seawater-associated bacterial communities are inherently variable over a scale of days due to changing environmental conditions (Powell et al., 2013; Magnesen et al., 2013; Kwan and Bolch, 2015). Furthermore, findings in Chapter 3 showed that replicate larval cultures having similar starting bacterial communities can develop different communities which later coincide with high mortality. Therefore, seawater and/or factors such as algal food (Dubert et al., 2015) and organic particulates (Magnesen et al., 2013) may represent important stimulation variations of microbial community structures and dynamics leading to the poor predictability of bacillary necrosis. In order to examine the link between the seawater associated bacterial communities and bacillary necrosis, this study developed, small-scale highly replicated experimental systems using 0.22 µm filtered seawater and a commercial algal food.

With the aid of higher number of replicate cultures, this study was carried out to compare the proteolytic activity and associated changes in the genetic composition of microbial communities associated with low and high larval mortality.

4.3 Materials and methods

4.3.1 Larval cultures

In commercial mussel hatcheries, the first week of larval rearing is typically a static culture system with a complete water exchange at 48 h intervals. As the majority of severe mortalities are during this early rearing phase, we used small-scale static

culture systems stocked at standard commercial larval concentration with mussel larvae at 4 dpf. Nineteen replicate cultures, the maximum number based on the availability of mussel larvae were established using 4 dpf blue mussel (*Mytilus galloprovincialis*) larvae sourced from Spring Bay Seafoods (SBS) commercial hatchery, Triabunna, Australia. Each culture contained 50 ml of 0.22 µm filter sterilised 35 ppt seawater in a transparent 60 ml flat bottom polystyrene container. Larvae were added to a final density of 10 larvae ml^{-1}. The container were gently aerated/mixed using 0.22 µm filtered air supply delivered to the bottom of container using sterile glass pipette, at about one bubble every 5 seconds as vigorous mixing can cause fatigued larvae (Helm et al., 2004).

The larval cultures were maintained at 22 °C with a total water change and algal food replenished every 48 h. Commercial microalgal paste *Isocrhysis* 1800 (Reed Mariculture Inc., California, USA, Appendix 6) was added to give a final algal concentration of 7.2×10^4 cells ml^{-1} (equivalent of 7.2×10^3 cells $larvae^{-1}$ as used in commercial mussel hatcheries; Appendix 1). The proportion of immotile larvae, an indication of moribund larvae (Chapman, 2012) and dead larvae was determined every 24 h by collection of larvae from the bottom of the container using a sterile disposable plastic pipette. Larvae collected were examined and counted under a dissecting microscope (Leica MZ12.5, × 40 and × 100 magnification, Germany); larvae showing active intestinal activity were recorded as immotile, whereas larvae with no evident intestinal activity were recorded as dead. After assessment, the sampled larvae (both live and dead) were returned to the culture container.

As this study focused on changes associated with increased mortality, all larval cultures were sampled (and the experiments terminated) when one or more larval cultures showed ≥50 % immotile larvae within a 24 h period. Cultures were grouped into low (less than 10 % mortality) and high mortality (≥50 % death in a 24 h duration).

4.3.2 Abundance of culturable and proteolytic bacteria

Seawater and larval sub-samples were collected from all high mortality cultures and and equivalent number of randomly selected low mortality cultures. Bacterial and *Vibrio* total viable count (TVC) were estimated using MA (Zobell, 1944) and TCBS (Oxoid) agar, respectively. Sub-samples of culture seawater (15 ml) containing larvae were gently removed using a sterile syringe, and pre-filtered though autoclave-sterilized 50 µm mesh (Allied Filter Fabrics, Hornsby, Australia) to separate larvae from culture seawater and the seawater was retained for TVC determinations. Larvae were then rinsed 3 times with autoclaved seawater, resuspended in 0.5 ml of sterile seawater, transferred to a sterile 1.5 ml centrifuge tube, and homogenised using a plastic micro-pestle. The retained culture seawater and larvae homogenates were both serially diluted to 10^{-7} in sterile seawater and plated onto MA and TCBS plates using an Advanced Instruments Autoplate® 4000 Spiral Plater (Advanced Instrument, Inc., Norwood, United States). For a single dilution series, 2 plates of the MA and TCBS showing 30-300 colonies after 48 h of incubation at 23 °C were counted and averaged to determine colony forming units (CFU) per ml of seawater and per larva.

The proportion of proteolytic and non-proteolytic bacteria was determined from MA spread plates from 2 randomly chosen high, and 2 low mortality cultures. For each culture, 90 to 100 colonies on the MA plates were randomly picked using sterile pipette tips and transferred onto double layered marine-milk agar plates made according to Sizemore and Stevenso (1970). Briefly, the bottom layer of milk agar (2 % dry milk powder, 1.5 % agar in 35 ppt seawater) was poured and allowed to cool and a second Marine Agar (Zobell, 1944) layer overlaid. Colonies transferred to marine-milk agar plates were incubated for 48 h at 23 °C. Colonies surrounded by a transparent zone (see Figure 4-6a) were considered to be extracellular protease producers and the proportion of proteolytic bacterial colonies recorded.

4.3.3 DNA extraction

The remaining 35 ml of culture was used for DNA extraction. Seawater and larvae were separated and prepared using the same methods described for bacterial plate count. Additionally, the culture seawater was passed though sterile 0.22 µm Supor® 200 polyethersulfone PES membrane filters (Pall Corp.,New York, United States). The homogenised larvae and culture seawater filters were stored in -20 °C until DNA extraction using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. A 2 cm^2 section of 0.22 µm filter was used for culture seawater extractions; 100 µl of the larval homogenate was used for larval DNA extraction.

4.3.4 Illumina sequencing of 16S rRNA gene

Microbial diversity profiling was carried out by Research and Testing Laboratories (Lubbock, Texas, USA) using the Illumina MiSeq platform. Pair-ended PCR amplification of the V3 hypervariable region of the 16S rRNA gene was carried out using primers 341F and 519R with 12 bp barcode tags. FASTQ files generated were merged using PEAR (Zhang et al., 2014) and were then trimmed to remove primer, barcode and adapter regions using an algorithm developed internally by Research and Testing (Lubbock, Texas, USA). The seed sequence for each cluster was then sorted by length and clustered with a 4% divergence cut-off to create centroid clusters. Clusters containing <2 sequences or sequences <100 bp in length were removed. Seed sequences were clustered again at a 4% divergence level using UPARSE (Edgar, 2010) to determine whether additional clusters could be detected. Consensus sequences from clusters were obtained using UPARSE (Edgar, 2013). Each consensus sequence and its clustered centroid of reads was analysed using UCHIME in the de novo mode to remove chimeras (Edgar et al., 2011). Each consensus sequence and its centroid cluster were denoised in UCHIME with base position quality scores of >30 acting as the denoising criterion. Sequence de-replication and OTU demarcation was performed in USEARCH and UPARSE to yield OTUs that were then aligned using MUSCLE (Edgar, 2004) and FastTree (Price et al., 2010) to infer approximate maximum likelihood phylogenetic trees. The OTUs were classified using Silva SSU database Release 128 (Quast et al., 2013). The 16S rRNA sequence read abundance was adjusted using the median number of 16S rRNA gene copies found in each taxon on a publically accessible database

rrnDB available at https://rrndb.umms.med.umich.edu/ (Stoddard et al., 2015) to improve estimates of microbial abundance.

4.3.5 Microbial community analysis

16S rRNA Illumina sequencing data for seawater (n=19) and larvae (n=19) were combined into a taxa-abundance matrix. 16S rRNA sequence data was organised and examined at phylum, class, order, family, genus, species and OTU level. The data were fourth-root transformed and a sample pairwise distance matrix calculated using Bray-Curtis dissimilarity coefficients. Community structure differences were examined using permutational multivariate ANOVA (PERMANOVA, p=0.05 at default settings, 9,999 permutations) and Principal Coordinates (PCO) in the Primer 6 + PERMANOVA package (version 6.1.12 and version 1.0.2; Primer-E, Ivybridge, UK). Bacterial taxa associated with mortality were also examined using linear discriminant analysis effect size (LEfSe) (Segata et al., 2011), available publicly online (https://huttenhower.sph.harvard.edu/galaxy/). The same dataset was used and analysed at default statistical setting (p=0.05 for non-parametric factorial Kruskal-Wallis (KW) sum-rank test and Wilcoxon-rank sum test) with default theshold on the logarithmic LDA score set at 2.0.

4.3.6 Proteolytic activity

Proteolytic activity of culture seawater sub-samples was determined using EnzChek BODIPY-FL protease assay kit (Catalogue No. E6638, Molecular Probes, Invitrogen, USA) with reagents prepared as per the manufacturer protocols. Analysis was carried

out in 200 µl reaction volumes using Greiner CELLSTAR 96 well clear flat bottom plates (Greiner Bio-One, Austria). Assay reactions consisted of 100 µl of reconstituted BODIPY-FL casein derivatives with 2 mM sodium azide, 50 µl of 10 mM Tris-HCL pH 7.8 and 50 µl of seawater. Activity was measured using a FLUOstar Omega fluorescent plate reader (BMG Labtech, Ortenberg, Germany) with excitation at 485 nm and emission detected at 520 nm. All samples were incubated at 25 °C for 48 h before reading fluorescence (maximum signal to background ration determined from preliminary analyses). All assays were performed in triplicate. A standard curve was generated ($y = 109033x - 2027.2$, $R^2 = 0.9805$, where y is fluorescence intensity, x is trypsin concentration in mg l^{-1}) using a 6 trypsin concentration series (ranging from 0.0016 to 1 mg l^{-1} in 10 mM Tris-HCL pH 7.8).

Data were subjected to one-way ANOVA, with comparisons of means using Tukey's Post-hoc test at p=0.05. Analysis for association of seawater proteolytic level and mortality were conducted using Fisher's Exact Test (p=0.05) as one or more cells in the contingency table had expected frequencies of five or less. All statistical tests were conducted in SAS Enterprise Guide v5.1 (SAS Institute Inc., Cary, NC, USA).

4.4 Results

4.4.1 Larval immotility and mortality assessment

At 24 h rearing, the larval populations in all 19 individual cultures maintained low levels (< 10 %) of immotility and mortality. However, at 48 h cultures 2, 9, 10 and 15, showed > 90 % immotility (Figure 4-1) and were significantly higher (see black bar with red outline, F=293.12, df 18, p<0.001) than the remaining 15 cultures which showed immotility levels ranging from 5 to 16 %. Examination of larvae for intestinal movement showed that in cultures 2 and 15 all immotile larvae were dead (98.8 % and 97.6 % mortality respectively, grey bar of Figure 4-1) whereas only 49.6 % and 77.6 % of larvae were dead in cultures 9 and 10. Mortality in all 4 cultures was significantly higher than the other 15 cultures (F=161.77, df 18, p<0.001) which exhibited ≤10 % mortality.

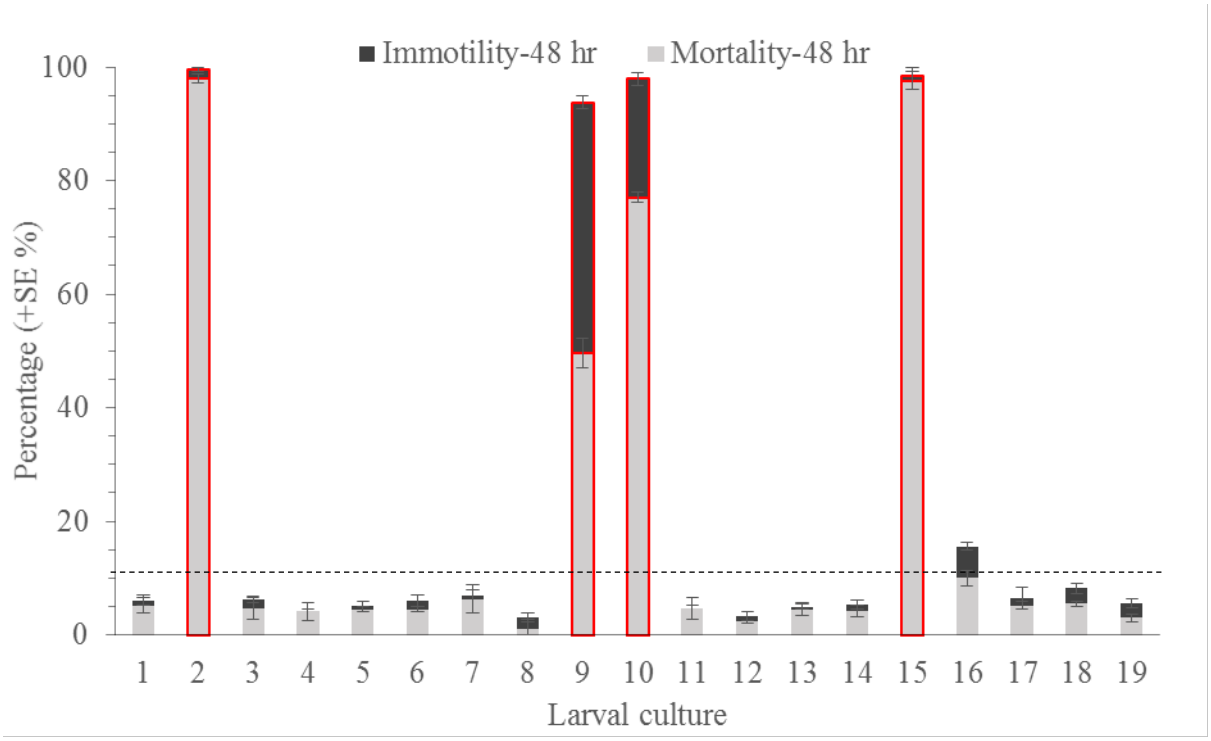


Figure 4-1 Percentage of immotile (black bar) and dead (grey bar) mussel larvae (±SE) for each of the 19 larval cultures 48 h of rearing. Dotted horizontal line denotes commercially acceptable mortality (10 %). Significant differences in larval immotility and mortality at 48 h are highlighted in red.

Total viable counts (TVC) of seawater in high mortality cultures ranged from 7.0 to 7.7 log CFU ml^{-1}, comparable to those of low mortality cultures. Except for low mortality culture 1 (TVC 5.4 log CFU $larva^{-1}$, TCBS representing total culturable *Vibrio*, 2.5 CFU $larva^{-1}$), TVC and TCBS of larvae from low and high mortality cultures were less than 3.2 and 1.3 log CFU $larvae^{-1}$, respectively. TCBS counts and TVC of seawater and larvae samples from 4 high mortality and 4 randomly sampled low mortality larval cultures (Figure 4-2) were not statistically different (TVCs, seawater F=0.04, df 1, p=0.8420, larvae F=0.66, df 1, p=0.4484; TCBS, F=1.33, df 1, p=0.2921, larvae F=0.99, df 1, p=0.3590).

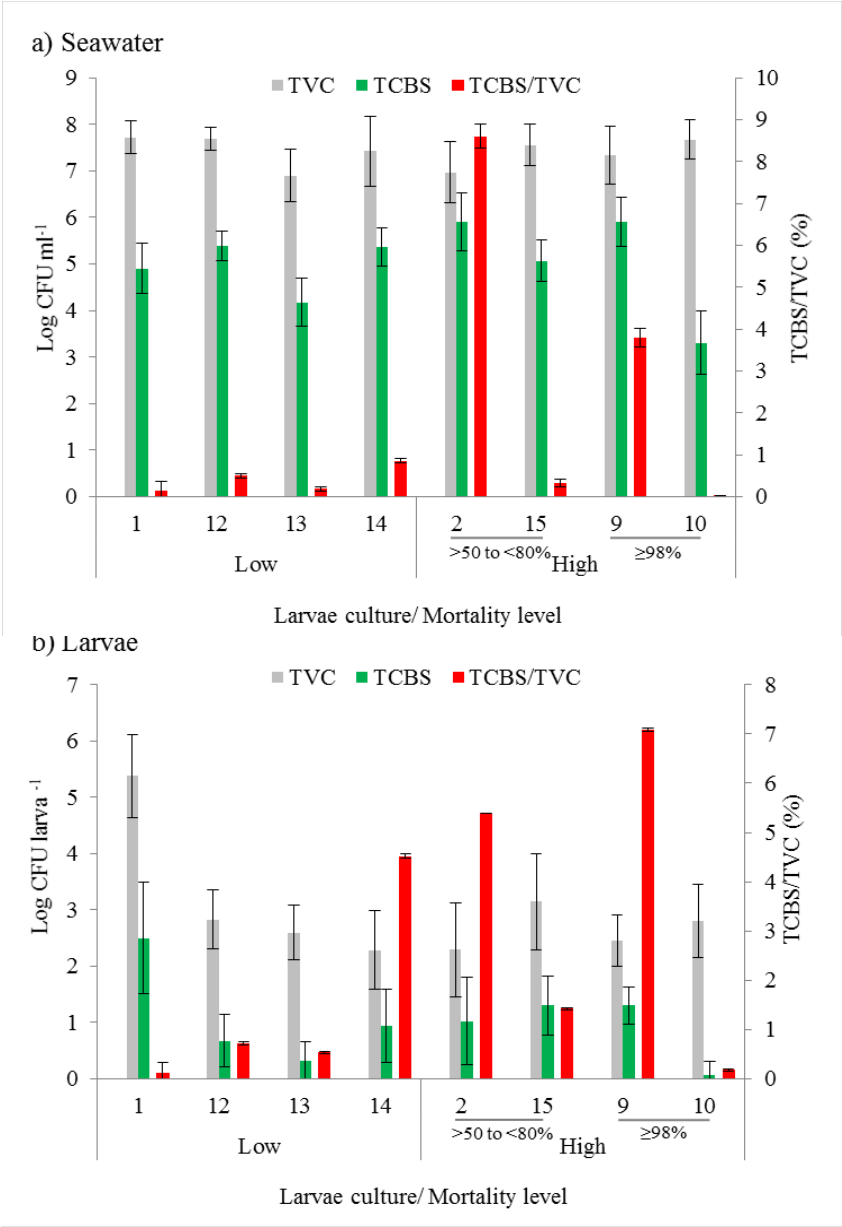


Figure 4-2 Plate count of total viable count (TVC) and total culturable *Vibrio* using MA and TCBS for a) seawater and b) larvae sampled from low mortality (culture 1, 12, 13 and 14) and high mortality larval cultures (cultures 2, 9, 10 and 15) at 48 h rearing.

The extent of presumptive *Vibrio* dominance in the seawater was generally reflected in the larvae. As observed in the seawater, higher predominance of *Vibrio* (>4.5 %) in larvae was also noted in the low mortality culture 14, and high mortality

cultures 2 and 9. Even though culture 9 larvae showed the highest TCBS to TVC ratio of 7.1 %, it represented a TCBS cell count of only 2×10^1 CFU larvae^{-1}.On the contrary, in culture 1 the highest *Vibrio* cell count of 3.2×10^2 CFU larvae^{-1} was observed, however this only accounted for 0.12 % of the TVC value.

The highest *Vibrio* relative abundance of 8.6 % (representing 8×10^5 CFU ml^{-1}) was observed in seawater of high mortality culture 2, which also had the highest mortality level of 98.8 %. However, patterns of seawater *Vibrio* dominance was not consistently associated with mortality. While higher *Vibrio* relative abundance in seawater and larvae was more frequently observed in high mortality cultures (Figure 4-2), *Vibrio* dominance was not statistically different between high and low mortality cultures (seawater F=1.33, df 1, p=0.2921; larvae F=0.99, df 1, p=0.3590)

4.4.2 Comparisons of bacterial communities

In total, 38 DNA samples (19 seawater and 19 larvae) were prepared for bacterial community analysis, of which 8 were associated with high mortality and 30 with low mortality. Comparison of bacterial communities using Principal Coordinates (PCO) analysis (or Multi-dimensional Scaling MDS in Appendix 5) indicate that larval associated bacterial communities in low mortality cultures were more similar to each other (clustered tightly) and less variable compared to communities associated with culture seawater (Figure 4-3). This contributed to the clearer separation of larval community associated with mortality (Figure 4-3b) when compared with the more variable communities found in seawater samples (Figure 4-3a).

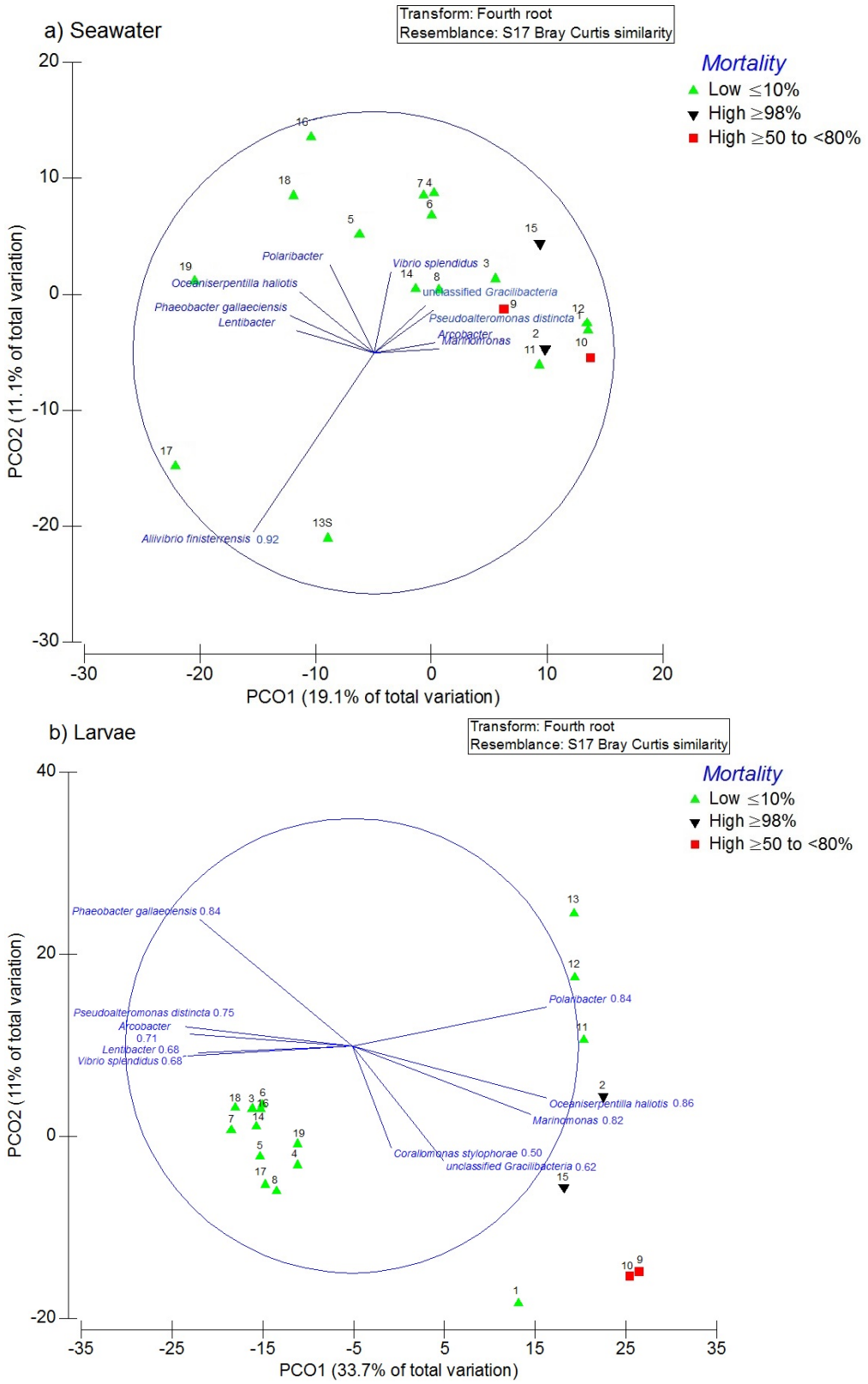

a) Seawater
Transform: Fourth root
Resemblance: S17 Bray Curtis similarity
Mortality
Low ≤10%
High ≥98%
High ≥50 to <80%
PCO2 (11.1% of total variation)
PCO1 (19.1% of total variation)
Polaribacter
Vibrio splendidus
unclassified Gracilibacteria
Oceaniserpentilla haliotis
Phaeobacter gallaeciensis
Lentibacter
Pseudoalteromonas distincta
Arcobacter
Marinomonas
Alivibrio finisterrensis 0.92
b) Larvae
Transform: Fourth root
Resemblance: S17 Bray Curtis similarity
Mortality
Low ≤10%
High ≥98%
High ≥50 to <80%
PCO2 (11% of total variation)
PCO1 (33.7% of total variation)
Phaeobacter gallaeciensis 0.84
Pseudoalteromonas distincta 0.75
Arcobacter 0.71
Lentibacter 0.68
Vibrio splendidus 0.68
Polaribacter 0.84
Oceaniserpentilla haliotis 0.86
Marinomonas 0.82
Coralomonas stylophorae 0.50
unclassified Gracilibacteria 0.62

Figure 4-3 Principal coordinate (PCO) plot of: a) seawater and b) larval bacterial communities associated with low (≤10%) and high (≥50 to <80%, and ≥98%) mortality. Each plot is vectored with 10 relatively most dominant bacterial taxa. Spearman's correlation (0 to 1, with the circle boundary representing the maximum score of 1) for each taxon is included indicating contribution to separation of community. Correlation of less than 0.5 is not shown.

Based on PERMANOVA analysis of Illumina MiSeq derived 16S rRNA gene taxa-abundance data, the larval bacterial communities associated with high mortality differed at all 7 taxonomic levels (Table 4-1). In contrast, bacterial communities associated with culture seawater in high mortality cultures differed only weakly at family level (p=0.0386).

Table 4-1 PERMANOVA analysis for bacterial community difference at 6 taxonomic levels for seawater and larvae samples associated with high (n=4) and low (n=15) mortality cultures.

		Phylum	Class	Order	Family	Genus	Species	OTU
Seawater	F	1.6491	1.8179	1.6233	**1.6817**	1.3742	1.2733	1.2482
	p	0.1316	0.0652	0.064	**0.0386**	0.0935	0.1287	0.1321
Larvae	F	**6.0583**	**4.419**	**6.3922**	**5.9228**	**4.2983**	**3.9048**	**3.7784**
	p	**0.006**	**0.0058**	**0.0009**	**0.0002**	**0.0007**	**0.0004**	**0.0002**

Larval bacterial community associated with high mortality (≥50 to <80 % and ≥98 %) were all displaced toward the lower right of the PCO plot (Figure 4-3b). High mortality cultures 9 and 10, which contained a mix of dead and dying larvae, clustered close together whereas culture 2 and 15 with ≥98 % mortality were slightly more separated from each other. The clustering of samples in relation to mortality level was observed for all taxonomic levels (Appendix 8) whereas clustering of high mortality cultures was not evident in seawater community samples (Appendix 9).

Interestingly, the larval communities observed in cultures 1, 11, 12 and 13 were also displaced to the right on the PCO1 axis, along with high mortality cultures (Figure 4-3b) yet did not show elevated immotility or mortality. The seawater communities of these cultures were also displaced to the right of the PCO1 with the high mortality cultures (Figure 4-3a).

The top 5 relatively most abundant sequence reads affiliated at genus levels accounted for over 95 % of the total reads of seawater and larval samples (Figure 4-4). The order of dominance of each taxon was comparable in seawater (Figure 4-4a) and larvae (Figure 4-4b) with sequences affiliated with *Pseudoalteromonas distincta* being the most dominant followed by *Arcobacter* sp., *Lentibacter* sp., *Marinomonas* sp., *Polaribacter* sp., unclassified *Gracilibacteria* and *V. splendidus*.

Notably, increased dominance of reads affiliated with *Polaribacter* sp., *Marionomonas* sp., and unclassified *Gracilibacteria* were observed in larvae from high mortality cultures, whereas reads affiliated with *Pseudoalteromonas distincta*, *Arcobacter* sp., *Lentibacter* sp. were more dominant in low mortality cultures.

The 2 levels of mortality (≥50 to <80 %, and ≥98 %) observed in cultures 2, 9, 10 and 15 were reflected in the compositions of bacteria. Cultures 9 and 10 suffering morbidity (immotile larvae and dead) contained a higher relative abundance of larval *Marinomonas* sp. affiliated reads (mean 10% versus 3%) and larval *Gracilibacteria* affiliated reads (10% versus 0.5%) compared to the mostly dead larval cultures 2 and 15. However, the relative abundance of seawater *Marinomonas* sp. and *Gracilibacteria* affiliated reads were variable across the 19 cultures regardless of mortality levels. Cultures 11, 12 and 13, which showed low mortality levels also had divergent larval bacterial communities with increased relative abundance of *Polaribacter* sp. but lower proportions of *Marinomonas* sp. and *Gracilibacteria* affiliated reads (see Figure 4-4a and b).

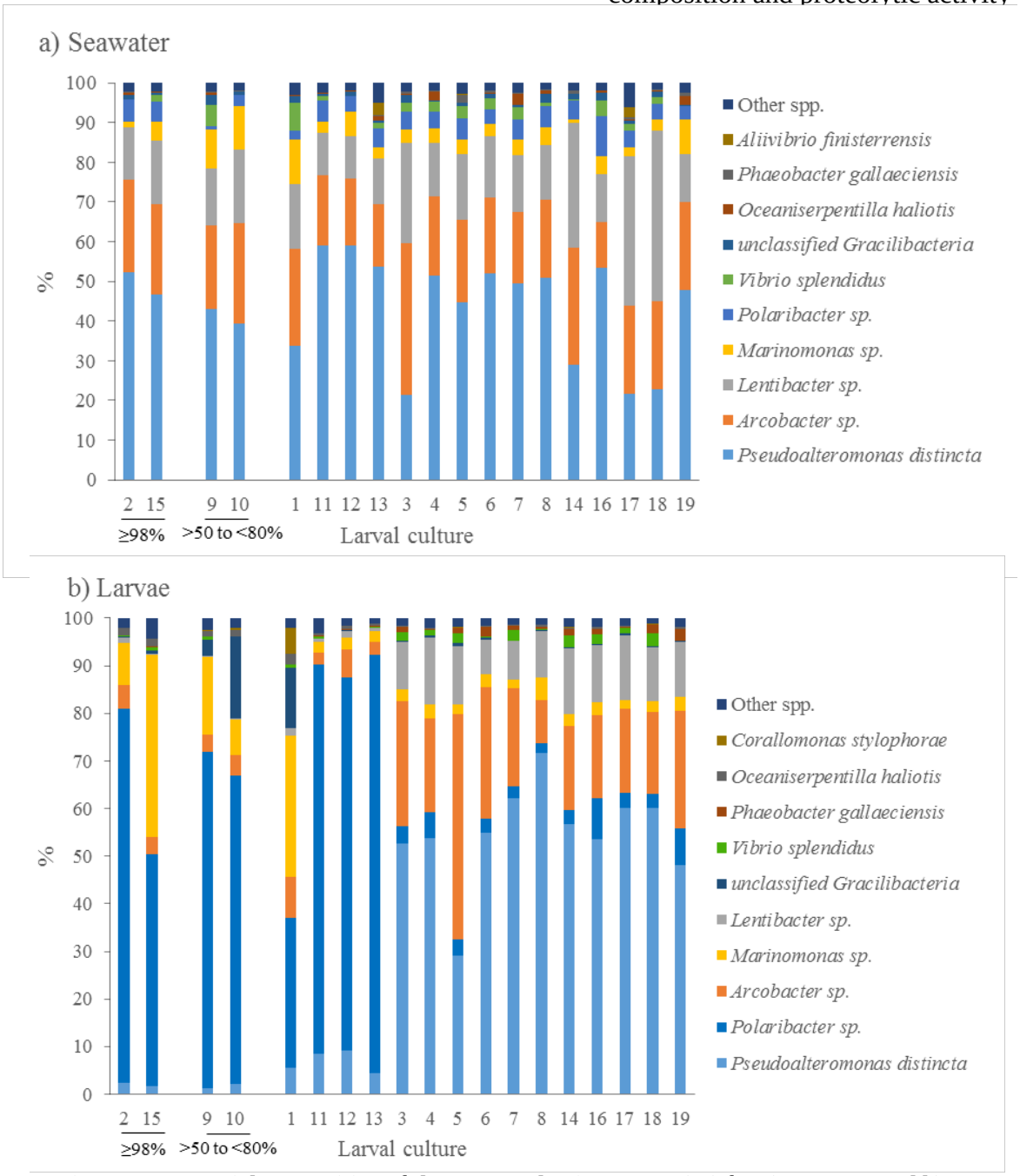


Figure 4-4 Bacterial composition of the 10 most dominant taxa (%) for a) seawater and b) larvae grouped according to high mortality (culture 2 and 15 ≥98 %; culture 9 and 10 ≥50 to <80%) and low mortality (≤10%) cultures. Culture 1, 11, 12 and 13 were grouped together as they distributed close to the high mortality culture in PCO (Fig.3b). Taxa (bottom to top) are in decreasing dominance. Other spp. in seawater and larvae, includes 272 and 168 bacterial taxa which in total accounted for 1.8% to 6% and 1.1% to 4.4% respectively.

Vector directions of bacterial taxa and Spearman correlations overlaying the PCO plots show that the 10 most dominant taxa in the larvae sample (Figure 4-3b) contribute substantially (Spearman correlation >0.6 to 0.9) to the changes in bacterial community associated with high mortality. Increased relative abundance reads affiliated with the 5 taxa (in decreasing order) of *Oceaniserpentilla haliotis*, *Polaribacter* sp., *Marinomonas* sp., unclassified *Gracilibacteria* and *Corallomonas stylophorae* were associated with high mortality cultures whereas the remaining 5 *Phaeobacter gallaeciensis*, *Pseudoalteromonas distincta*, *V. splendidus*, *Arcobacter* sp. and *Lentibacter* sp. were associated with low mortality cultures. In seawater, *Marinomonas* sp. and unclassified *Gracilibacteria* affiliated reads both of which showed low Spearman's correlation of <0.3 show weak association with seawater community of high mortality. *Phaeobacter gallaenciensis* (0.47), and *V. splendidus* (0.45) in seawater were more associated with low mortality cultures.

Given the significant association of larval bacterial community with mortality (PERMANOVA $p<0.05$, Table 4-1), linear discriminant analysis effect size (LEfSe) focusing on larval community data detected over 40 and 20 bacterial taxa (at LDA score greater than 2) with statistically different abundance in high and low mortality cultures respectively (see Appendix 10). Ranking of linear discriminant analysis (LDA) scores of these dominant taxa shows the relatively most abundant reads associated with high and low mortality were affiliated with the unclassified *Gracilibacteria* with a LDA score of -4.1, and *Pseudoalteromonas distincta* with a score of 4.6 respectively (Figure 4-5). *Gracilibacteria* were overrepresented in high mortality cultures, with mean 6.5±2.7 % versus 1.0±0.8 %. *Marinomonas* was

detected with lower LDA score of -3.5 but showed relatively more abundant reads at 18.5±6.8 % versus 4.1±1.8 %. *Pseudoalteromonas distincta*, *Phaeobacter gallaeciensis* and *V. splendidus* affiliated reads were relatively more abundant in low mortality at 40.5±6.0 % versus 4±0.9 %, 2.7±0.3 % versus 0.3±0.1% and 0.7±0.8 % versus 0.4±0.2 % respectively.

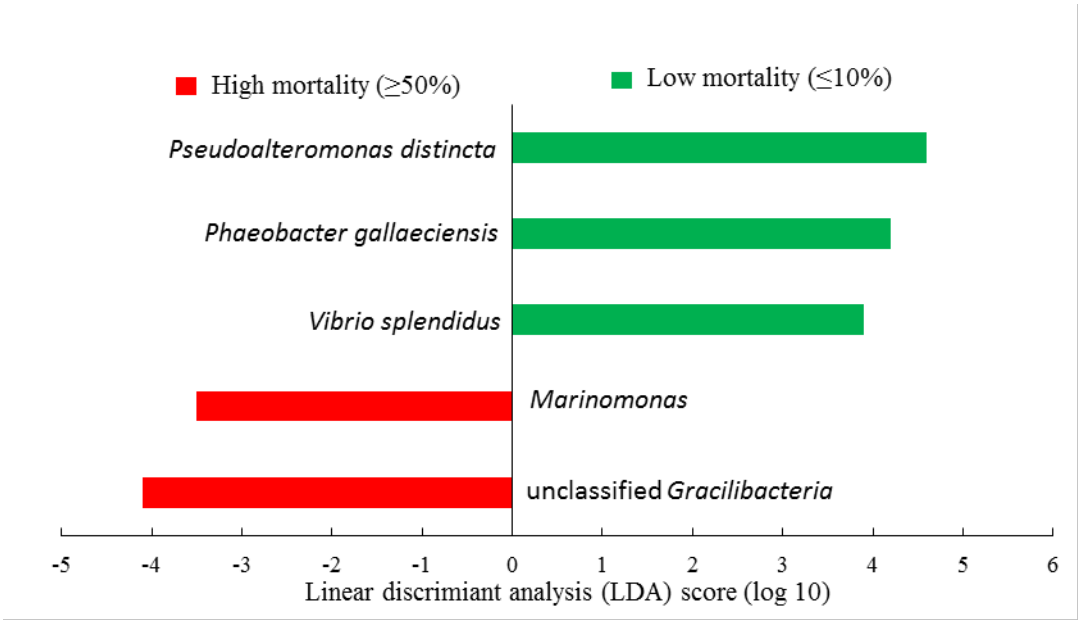


Figure 4-5 Histogram of linear discriminant analysis (LDA) score for statistical and differentially abundant bacterial taxa in the larvae of low and high mortality cultures. The LDA scores (2 regarded as minimum and 5 high (Segata et al., 2011) represents the degree of consistent difference in relative abundance between the two mortality classes. Taxa with the highest scores explain the greatest difference communities between the 2 mortality classes.

4.4.3 Proteolytic activity

Screening using marine- milk agar detected a significantly higher proportion of culturable proteolytic bacteria in seawater samples from high mortality compared with low mortality cultures (99.0% versus 14.3%, Chi-square 123.9, df 1, p<0.0001). Interestingly, the opposite was observed in larvae samples where high proportion of proteolytic bacteria were detected in low mortality compared with high mortality cultures (58.8% versus 8.4%, chi-square 52.86, df 1, p<0.0001).

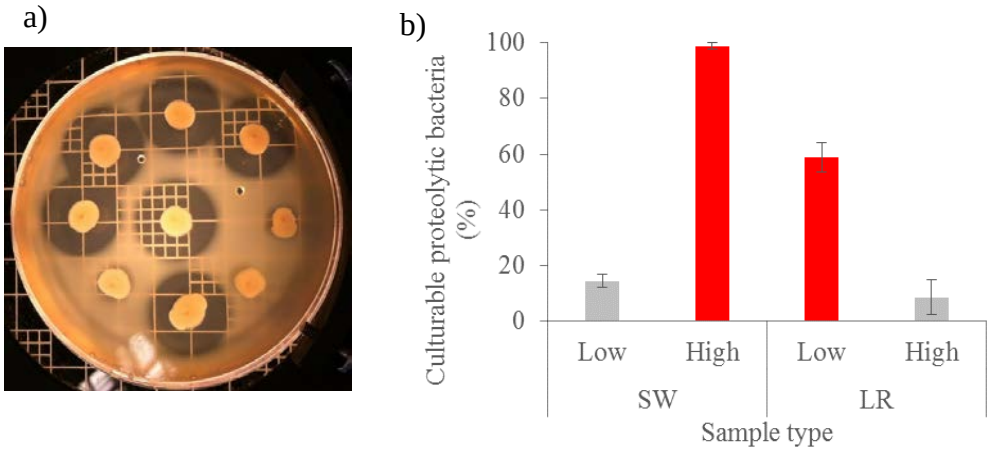


Figure 4-6 Proteolytic activity of bacteria from high and low mortality cultures. (a) An example of proteolytic (clear zones) and non-proteolytic colonies (no clear zone) on milk agar. (b) Percentage of proteolytic bacteria in low (culture 12 and 13) and high mortality cultures (2 and 9) for seawater (SW) and larvae (LR). Red bar indicates significant difference using Chi-square (p=0.05).

Pair-wise one-way ANOVA shows total proteolytic activity (TPA) in rearing seawater of high mortality culture 2, 9 and 10 were statistically higher (0.66±0.06 mgl^{-1} trypsin equivalent, Figure 4-7) compared with the 15 low-mortality cultures (0.33±0.01 mgl^{-1} trypsin equivalent). Interestingly, TPA of culture 15 was

relatively low and therefore not statistically different with those from low mortality cultures. Contingency analysis showed a significant association between total proteolytic activity (3 high and 15 low levels) and larval mortality (4 high and 15 low levels; Fischer's test; p=0.0041).

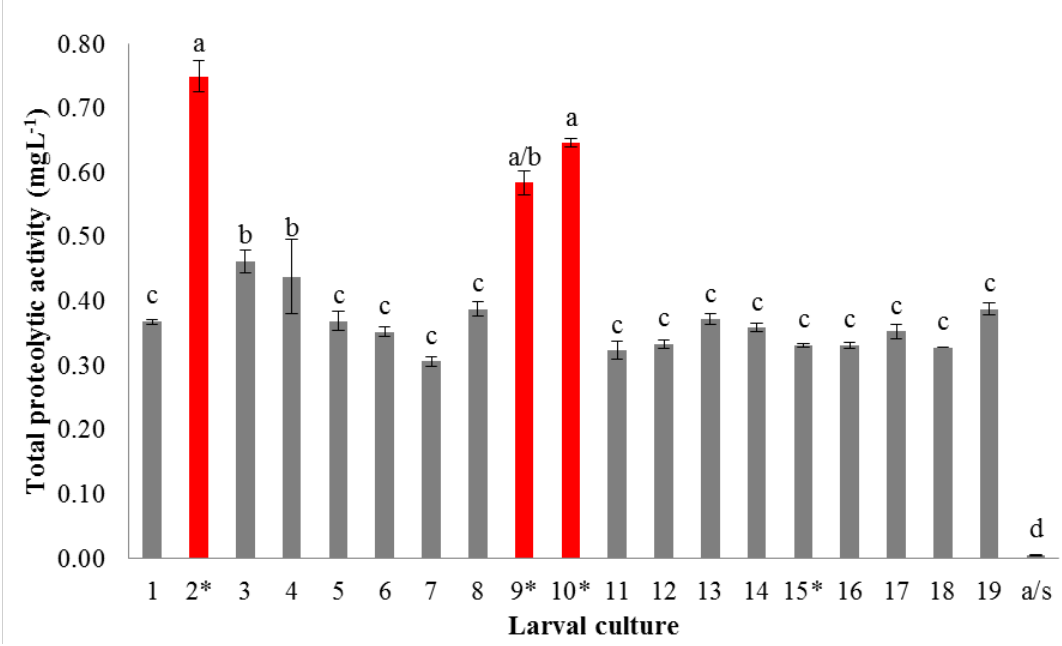


Figure 4-7 Total proteolytic activity (TPA, equivalent mg trypsin L^{-1} seawater, mean ± SE) of seawater sampled from larval cultures 1 to 19 analysed using protease fluorescence assay. Asterisk indicates cultures suffering high mortality levels (culture 2 and 15, ≥98 %; culture 9 and 10, ≥50 to 80 %). Different letters indicate significantly different levels using one way ANOVA (p=0.05) and the red bars indicate significantly higher levels of TPA. a/s denotes aged seawater.

4.5 Discussion

Earlier experiments led to the hypothesis that seawater is an important source of microbial variation accounting for the sporadic nature of bacillary necrosis. This led us to anticipate control of seawater quality employed in this study would produce more consistent larval culture bacterial communities and better predictability of bacillary necrosis. This study however observed bacillary necrosis remain sporadic in individual larval cultures, suggesting variability in bacillary necrosis under these circumstances could still be influenced by small/difficult to control sources of variations. The 0.22 µm filtered seawater and commercial algal paste were not determined to be free of bacteria and it is also possible minimal variations in the starting materials can rapidly develop and influence microbial dynamic in the larval cultures.

Bacterial community analyses based on high throughput amplicon sequencing or metagenetic show mass mortalities in this study were more clearly associated with changes in larval bacterial communities compared with seawater. Interestingly, the use of sterile seawater did not result in as clear association of seawater bacterial community with mortality as intended. Patterns of PCO plots indicates that seawater communities overall are more variable, resulting in poorer separation of high and low mortality samples. The use of 0.22 µm filtration would have removed the majority of bacteria but the microbially depleted seawater rapidly receives and reflects bacterial community inoculum from the larvae resulting in the similar seawater-larval bacterial communities (compare Figure 4-3a and 4b). This lack of separation of

seawater community as opposed to chapter 3, can be attributed to this study only considering community at one time point therefore limiting the ability to discern community shifts. This is particularly relevant because seawater community in larval culture is inherently variable (Powell et al., 2013) in comparison with larvae (chapter 3).

The sporadic mass mortality in the 4 cultures is linked with a similar group of larval-associated bacteria, indicating that after onset of mortality, similar larval bacterial communities developed during the progression of the disease. The selective pressure/s driving these changes is difficult to pinpoint but that the larval bacterial community is known to change during larval growth. The relatively short bivalve larval stage (typically 2-3 weeks) means larval growth is rapid with changing physiological conditions that is reflected in successional changes of larval associated bacteria during the period of larval culture (Sandaa et al., 2003; Godoy et al., 2011; Sun et al., 2016). Using high throughput sequencing, Sun et al. (2016) demonstrated distinctive bacterial composition in fertilisation, trocophore, D-shape, umbo larvae and juvenile stage, indicating larval bacterial community evolves with growth and possibly does not become stabilised until spat stage. This suggests that larval associated communities can be unstable by nature and vulnerable to subtle disturbances experienced in this highly-controlled larval cultures, leading to abnormal shifts in community. Together with information in Chapter 3 that shows changes in larval community preceded mortality, the changes of bacterial compositions observed in this study may well be linked with bacillary necrosis.

Analysis based on taxon-relative abundance shows that community shifts comprise a multitude of bacteria some of which shifted in relative abundance to a greater extent than other bacteria. This together with the low number of high mortality replicates led to difficulty in using LEfSE to confidently resolve important bacterial taxa in relation to larval survival. This is reflected in the detection of high number (over 50, see Appendix 10) of bacterial taxa with weak to moderate LDA scores. Therefore, this study focused on the dominant bacterial taxa determined to be significant by LEfSe.

Several dominant taxa primarily *Polaribacter*, *Gracilibacteria* and *Marinomonas* are most clearly associated with high mortality (cultures 2, 15, 9 and 10). Their difference in relative abundance supported the observed two high mortality levels (50% dead with remaining 45% moribund, and near complete mortality) indicative of 2 progression stages of disease: morbidity and complete mortality. Additionally, several low mortality cultures (particularly culture 1, 11, 12 and 13) were similar to cultures showing high mortality, particularly due to an increased proportion of *Polaribacter*. This bacterial genus was most dominant (mean 69 ± 12 %) in these presumably early diseases symptoms free larvae and decreased in dominance in morbidity and complete mortality cultures (mean 68.5 % and 63.5 % respectively). *Polaribacter* therefore remained the most dominant taxa in the four high mortality larval cultures but morbidity was characterised by increased proportion of seawater *Marinomonas* and an unclassified bacteria belonging to candidate phylum *Gracilibacteria* in larvae, each accounted for over 10 % of total bacterial community. Dominance of *Gracilibacteria* and *Marinomonas* characterised morbid larval

cultures as shown by vectored PCO plot (Figure 4-3b) and the bacterial composition graph (Figure 4-4b). These two bacterial genera are detected by LEfSe to be statistically significant with moderate LDA scores.

Polaribacter belongs to the phylum *Bacteroidetes* and is abundant in marine systems and plays important role in degradation of high molecular weight organic matter. Growth of *Polaribacter* has been shown to be stimulated by blooms of phytoplankton due to it being an efficient consumer of algal materials (Xing et al., 2015). Therefore, it can be assumed that the use of algal paste in this study may have contributed to its dominance. However, the substantially higher dominance (almost 10 fold) in larvae from high mortality cultures is unusual and may be a response to release of organic matter from sick but surviving larvae. Due to the combination of insufficient sequence variation and short sequence reads (i.e 170 bp of the approximate 1500 bp) of the partial 16S rRNA gene, this study could not determine which species caused successional changes in the dominance. Studies show that *Polaribacter* species even with 98 % 16S rRNA gene sequence similarity have quite different genomes and thus may have different capacities for polysaccharide and protein degradation (Gomez-Pereira et al., 2012; Xing et al., 2015) suggestive of potentially variable interactions with marine hosts. Further studies primarily to characterise the diversity of *Polaribacter* and interaction with larvae such as using challenge bioassays are needed to confirm the linkage between *Polaribacter* and larval mortality.

Association of *Marinomonas* with mass mortality in bivalve larvae has never been

reported. It is an aerobic heterotroph that can be isolated from hatchery seawater and oysters (Pujalte et al., 1999) and also healthy halibut larvae (Bjornsdottir et al., 2009). However, it is interesting that this study and several authors (Griffiths et al., 2001; Jensen et al., 2002; Jensen et al., 2004; Lauzon et al., 2010) isolated *Marinomonas* along with *Pseudomonas, Pseudoalteromonas* and *Vibrio* from marine larval culture systems indicating they occupy a similar ecological niche. Numerous strains from this niche are associated with mortality in reared bivalves (Garland et al., 1983; Elston, 1993; Torkildsen et al., 2005). Members of the genus *Marinomonas* such as *M. arctica* (Yoo and Park, 2016) and *M. blandensis* (Chimetto et al., 2011) have been reported to possess extracellular serine protease activity. Strains of this species can also hydrolyse casein in milk agar as detected in this study but we did not identify the proteolytic isolates recovered from this study.

A group of uncharacterised bacteria belonged to the candidate phylum *Gracilibacteria* was discovered. In this study, members of this group was found to be significantly more dominant in moribund larvae. *Gracilibacteria* belong to the super-phylum named *Patescibacteria*, containing metabolically reduced bacteria with small genomes (<1 Mbp) and presumably small cell size. There appears to be neither information on cell size nor surface structures of *Gracilibacteria,* both which are pertinent in predicting passage through the 0.22 µm filters. However, *Parcubacteria* to which *Gracilibacteria* is related distantly to, were isolated from a 0.22 µm filter (Nelson and Stegen, 2015) despite its presumably small cell size inferred from its comparably small genome size. Given the limited information, it is therefore difficult to predict whether the higher relative abundance of *Gracilibacteria* in diseased

larval cultures originated from the seawater bacterial communities. Rinke et al. (2013) showed *Gracilibacteria* possesses a novel recoding of the stop codon UGA for glycine possibly reflecting an adaptation due to small genome size. A comparative genomic study carried on a related phylum *Parcubacteria* (OD1) revealed gene sets for biosynthesis of various essential organic compounds have been partly or entirely lost (Nelson and Stegen, 2015), indicative of highly specialised niche and/or a parasitic or symbiotic lifestyle. Their increased relative abundance may be due to changes in host microorganisms (such as bacteria, virus or fungus) associated with high level of morbidity in larvae.

The significant association of dominant *Pseudoalteromonas* and *Vibrio* with low mortality observed in this study has a mixed agreement with published studies. It is worthy of mention the lack of *Vibrio* affiliated sequence reads in mortality culture samples is not likely a result of primer bias. The choice of 341F and/or 519R has been routinely used and detected sequences belonged to *Vibrio* in various environment/species: Atlantic salmon (Zarkasi et al., 2016), Atlantic halibut (Jensen et al., 2004), fugu fish (Yang et al., 2007), kuruma shrimp (Liu et al., 2010b) and turbot (Cerda-Cuellar and Blanch, 2002). In fact, a study by Aravindraja et al. (2013) using similar primers (341F and 518R) and Illumina sequencing platform detected *Vibrio* of up to 30 % of total sequences in marine sediments but less than 1 % in seawater and seagrass, further supporting that the low sequence reads affiliated with *Vibrio* in this study is a true representation of bacterial community.

Pseudoalteromonas dominance of healthy larvae agrees with Sandaa et al. (2003) where the authors demonstrated persistence and dominance of *Pseudoalteromonas* in the early stage of scallop larval cultures. In the contrary, Sun et al. (2016) showed mass mortality in scallop larvae showed overwhelming succession from over 80 % of *Pseudomonas* in normal larvae to 56 % of *Pseudoalteromonas* of total bacteria in morbid larvae, a level interestingly comparable to our study. Within *Vibrionaceae, V. splendidus* are the most frequently implicated bacteria associated with mass mortality in larval cultures (Sugumar et al., 1998b; Lambert et al., 1999; Le Roux et al., 2002; Gay et al., 2004b). However, a study examining *Vibrio* dynamic in mass mortality of adult oyster demonstrated, of the bacterial species belonging to the *V. splendidus* group, *V. splendidus* and *V. gigantis* strains are the most dominant in normal oyster but are successively replaced by another closely related species *V. crassostreae* at the onset of mortality (Lemire et al., 2015). Additionally, *V. splendidus* related genotypes are the dominant culturable *Vibrio* in farmed mussel (Oden et al., 2016) and under normal non-disease conditions in mussel hatchery cultures (Kwan and Bolch, 2015) indicating that not all strains are virulent or have similar disease interactions. The mixed agreement of association of *Vibrio splendidus* related bacteria with bacillary necrosis could be to do with the high rate of horizontal gene transfers (Wildschutte et al., 2010) that may have the potential to result in acquisition or loss of virulence genes. However, it is not known whether important virulence factors encoded by the metalloprotease (*vsm*) and major outer membrane protein gene (*ompU*) can be horizontally transferred between Vibrio bacteria.

Milk marine agar screening compared a limited number of samples (i.e low mortality culture 12 and 13, with high mortality culture 2 and 9). However, both of the low mortality cultures actually showed a close aggregation of both larval and seawater communities with the four high mortality (see culture 2, 9, 10 and 15, Figure 4-3) suggestive of early developmental stage of mortality. Interestingly, the milk marine agar examination of the low mortality cultures showed a higher proportion (almost 6 times more, Figure 4-6) of proteolytic bacteria in larvae compared with the high mortality cultures. The pattern of higher relative abundance of proteolytic bacteria however was the opposite, i.e almost 9 times more in seawater of the high mortality culture as supported by the protease fluorescence assay (Figure 4-7). The preliminary observation of higher proportions of proteolytic bacteria in larvae at potentially early disease stage requires more investigation to demonstrate if bacterial proteolytic activity develop in the larvae before release into the seawater.

Laboratory-based larval challenge bioassays demonstrated that proteases of pathogenic bacteria when administered into seawater are capable of impairing crucial larval functions, resulting in tissue necrosis and ultimately death (Labreuche et al., 2010; Saulnier et al., 2010). This suggests the elevated proteolytic levels detected here could have roles in the death of larvae. As detected in this study, many bacteria such as those affiliated with *Pseudoalteromonas distincta* (Ivanova et al., 2004), various species of *Polaribacter* (Nedashkovskaya et al., 2005; Hyun et al., 2014), *Marinomonas* and *Vibrio splendidus* (Saulnier et al., 2010) are a common protease producers in seawater environments. An important question now is whether the protease/s are produced from a specific strain or a group of genetically diverse

but protease producing bacteria. The latter (on the assumption that increased proteolytic activity can result in larval mortality) would suggest bacillary necrosis is a result of community-wide activity, an idea that would challenge the current understanding of bacillary necrosis that is based on single pathogen–host interactions. In fact, a study by Lemire et al. (2015) has provided evidence that mass mortality in farmed oysters is caused by a diverse group of *Vibrio* which share a common virulence gene conferring infective capacity. A sensible follow-up study would be to employ a similar assay to monitor changes of proteolytic activity across a time series leading up the mortality in larvae culture to better correlate protease production between bacterial community and mass mortality.

Protease production by *Vibrio* is regulated by quorum sensing (De Decker et al., 2013) and avirulent bacterial species can produce similar quorum sensing molecules resulting in up-regulation of protease production in virulent strains. This indicates production of proteolytic enzymes in the larval culture is complex due to involvement of avirulent strains, and can switched on when the right taxa combinations are present and above threshold concentrations. It could be for this reason that seawater in high mortality culture 15 was not observed to have a higher proteolytic activity. The bacterial community of culture 15 was slightly different to those of culture 2 (see PCO at Figure 4-3a) due to detection of *Profundimonas* at 3%, which was not detected in the 3 high mortality cultures.

This study shows that variation in mortality are difficult to control and predict even at 50 ml culture scale, suggesting studies of this nature to produce balance datasets

amenable for hypothesis testing is challenging. Recommendation for similar work in the future should consider increasing substantially the number of replicate culture (e.g. to 100 units) as this would capture wider variation (such as more stages of disease) with higher numbers of replicates.

4.6 Conclusion

The study shows that variation of bacillary necrosis in terms of its occurrence and rate of progression is difficult to control and presumably is also influenced by factors other than seawater such as bacterial communities associated with the larvae themselves. Nevertheless, the highly controlled nature of this larval rearing experiment successfully observed the unpredictable timing and scale of mortality is consistently linked with a similar group of bacteria, indicative that after onset of mortality, the similar bacterial communities developed during the progression. Dominance of *Gracilibacteria* and *Marinomonas* characterised morbid larval cultures but due to insufficient sequence variation of the examined V3 region of 16S rRNA gene, genotypes driving the increased dominance cannot be described. This study shows that bacillary necrosis involves subtle variations that can rapidly lead to divergence of community at the times of disease. In addition to the community data, the positive correlation of proteolytic activity in rearing seawater, with mass mortality is novel, warranting more studies to further understand role/s of elevated proteolytic activity in seawater in bacillary necrosis.

Chapter 5 *Pseudoalteromonas* sp. : an opportunistic pathogen of Australian blue mussel (*Mytilus galloprovincialis* Lamarck) larvae causing velar deformation and detachment

5.1 Abstract

In this study, 10 bacterial colonies on marine agar (MA) were randomly isolated during a mass mortality event in laboratory larval cultures, and screened for virulence using mussel larval challenge bioassays. Thiosulfate-citrate-bile salts-sucrose (TCBS) agar however did not present any colonies. Assays showed that Log 7 cells ml^{-1} of the bacterial isolates in 1 µm filtered seawater were capable of causing 55±3 % and 52±8 % mortality 2 d post challenge (dpc) in 2 and 22 d post fertilisation (dpf) larvae respectively. The LC_{50} (2 dpc) of 6 dpf larvae, was lower when assays used 0.22 µm filtered sterile seawater ($3.2±2.1 \times 10^4$ cells ml^{-1}) than when using 1 µm filtered seawater ($2.2±1.6\times 10^6$ cells ml^{-1}). In challenge experiments with 22 dpf larvae, increased mortality was observed after 3 dpc (84±5 %) associated with an unintentional increase of challenge assay temperature from 22.6 to 24.2 °C. Challenges using 6 dpf larvae and bacterial isolates subjected to repetitive sub-culture resulted in significant reduction of 2 dpc cumulative mortality to a level equal to no bacteria controls, whereas assay with cultures derived from cryopreserved isolates retained virulence. Comparison of 16S rRNA gene sequences showed that all 10 isolates were identical and belonged to the genus *Pseudoalteromonas*, allied with a non-pigmented sub-cluster and closely related to *P. carrageenovora* at 99.1 %

similarity. Histopathological examinations identified early, mid, severe, completion and post mortem stage of disease development across 3 d characterised by cleavage and detachment of velum without direct invasion of the velar tissue by bacterial cells indicative of destruction mediated by a proteolytic toxin. However, marine-milk agar and azocasein assays detected only limited proteolytic activity of the *Pseudoalteromonas* isolates. Whilst more research is necessary, this study demonstrates for the first time a strain of *Pseudoalteromonas* can negatively implicate mussel larval cultures.

5.2 Introduction

The Australian mussel aquaculture industry is based on blue mussel *Mytilus galloprovincialis.* In 2013/2014 recorded production of 3237 tonnes, worth almost US $9.6 million (Savage and Hobsbawn, 2015). Australia imports mussels equivalent to 35 % of local production whilst neighbouring New Zealand in 2013 exported a US $120 million worth of farmed Greenshell mussels (*Perna canaliculus*) (Skirtun et al., 2013; Seafood New Zealand, 2014). This data suggests there is considerable potential for increased production of mussels for both the local and export markets.

Consistent commercial production of hatchery reared mussel spat is important to provide a reliable supply for the adult grow out phase. However, consistent larval production has been hampered by sporadic, rapidly progressing larval mortalities, mostly associated with bacterial causes (Anguiano-Beltran et al., 2004; Kesarcodi-Watson et al., 2009a). The identity and nature of bacterial diseases causing mussel mortality are not well described but to date most studies implicate species of *Vibrio* (*Vibrio alginolyticus*, *V.coralliilyticus*, and *V. splendidus* related strains) as the

most common pathogenic bacteria of mussel larvae (Anguiano-Beltran et al., 2004; Kesarcodi-Watson et al., 2009a). The view that *Vibrio* spp. are important shellfish pathogen is largely derived from challenge bioassays that show lethality of *Vibrio* isolated from diseased larval cultures. It is however yet to be established if *Vibrio* are the direct or indirect cause of disease. Nevertheless, a number of studies have also implicated a range of other bacteria such as *Cytophaga*-like bacteria (CLB) (Dungan et al., 1989), *Alteromonas* and *Pseudomonas* species (Lodeiros et al., 1987), *Roseobacter* (Boettcher et al., 2000), and *Halomonas* (Rojas et al., 2009) as causes of disease in bivalve larval cultures.

As a crucial step to developing efficient disease management strategies, it is important that the range of possible pathogens are identified. This study describes a novel, *Pseudoalteromonas* isolate that can be pathogenic to blue mussel larvae. Virulence to mussel larvae is confirmed using larval challenge bioassays, and the progression of the disease is described in experimentally infected larvae.

5.3 Methods

5.3.1 Isolation, storage and preparation of bacteria

Bacteria from larvae and seawater of a laboratory mussel larvae culture during onset of larval morbidity where >90 % of larvae became immotile were numerated using MA (Difco) and TCBS (Oxoid) agar. The moribund larvae were collected by pipette, gently rinsed with sterile seawater to remove loosely attached bacteria and detritus, and homogenised in a 1.5 ml tube using a micro-tube pestle. The homogenate and seawater samples were serially diluted to 10^{-7} using autoclaved seawater and spread

plated in duplicates using an Advanced Instruments Autoplate® 4000 Spiral Plater (Advanced Instrument, Inc., Norwood, United States). After 48 h incubation at 23 °C, plates containing 30 to 300 colonies were counted and averaged to determine colony forming units (CFU) per ml of seawater and per larva. A total of 10 bacterial colonies were randomly isolated from both seawater and larvae spread plates of the highest dilution , re-streaked to ensure purity, and stored at -80 °C in Tryptone Soy Broth (Oxoid, Australia) containing 20 % (vol/vol) glycerol as cryoprotectant.

5.3.2 Bacteria larval challenge bioassay

Mussel larvae were produced in the laboratory by thermally inducing spawning of matured female and male broodstock, sourced from a marine hatchery at Spring Bay (Triabunna, Australia). Larvae were raised in 1 µm filtered 35 ppt seawater maintained at 21-22 °C and fed daily with 3 x 10 cells $larva^{-1}$ algal culture *Isochrysis* sp. clone *T. iso* until used in challenge assays at 2, 6, 7 and 22 dpf. The algal cultures were determined to be free of culturable bacteria using MA. Details of the larval assays carried out are summarised in Table 5-1. Bacterial isolates were cultured in 5 ml of Marine Broth (MB, Difco) for 36 h at 25 °C in 25 ml McCartney bottles shaken at 170 rpm. An aliquot of 0.8 ml of culture suspension was transferred into 1.5 ml Eppendorf tubes and centrifuged at 3200 × *g* at 22 °C for 10 min. The supernatants were discarded, pelleted cells re-suspended and washed twice using 0.8 ml autoclaved sterile seawater. Bacterial cells were re-suspended in 0.22 µm or 1µm filtered seawater, the cell density determined using a haemocytometer, and the cell density was adjusted to Log 3, 5 or 7 cells ml^{-1} for bioassays.

Table 5-1 Detail of bioassays carried out using mussel larvae of varying age as dpf.

	Biossay 1	Biossay 2	Biossay 3	Biossay 4
Isolate (n)	T8H1 to T8H10 (10)	T8H10 (1)	T8H10 (1)	T8H10 (1)
Challenge concentration (Log cell ml^{-1})	7	7	3, 5, 7	7
Seawater	Non-sterile (1 µm)	Non-sterile (1 µm)	Non sterile (1 µm) and sterile (0.22 µm)	Sterile (0.22 µm)
Larva (dpf)	2	22	6	7
Larval density (ml^{-1})	250	25-30	25-30	20
Challenge duration (d)	4	3	2	3
Replicate	3	6	6	1
Temperature range (°C)	21.6-22.3	21.6-24.2	21.0-22.3	20.9-21.6

Preliminary virulence screening of 10 isolates (herein identified as T8H1 toT8H10) was conducted using a bioassay similar to that of Kesarcodi-Watson et al. (2009a) but carried out in 96 well plates. Each well contained 50 larvae in 200 µl (250 larvae ml^{-1}) of 1 µm filtered 35 ppt seawater with no algal food added. Bacterial suspensions were aliquoted to achieve final assay well concentration of 10^7 cells ml^{-1}. Triplicate bioassays were carried out for each bacterial isolate and larvae were challenged for 4 d. A positive control was included in assays which contained a known virulent strain of *Vibrio tubiashii* (strain 09/2885-1) obtained from Animal Health Laboratories (Launceston, Australia). Negative controls received 0.22 µm filtered sterile seawater.

Bioassays were repeated using the representative isolate T8H10 with 22 dpf larvae in 12 well plates at 30 larvae ml^{-1} for 3 d. Each well contained final assay concentrations of 10^7 cells ml^{-1} and was replicated 6 times. Preliminary assays showed that isolate T8H10 displayed reduced virulence after repeated sub-culture on Marine Agar (MA, Difco), therefore larval assay experiments were carried out to compare isolates subjected to 5 rounds of sub-cultures (total of 2 weeks between each sub-culturing, plates incubated at 25 °C for 36 h and then stored in 4 °C) with isolates recovered from cryopreserved stocks and subjected to 2 rounds of subcultures. This experiment was carried out using 6 dpf larvae at 20 larvae ml^{-1} in 12 well plates. Isolate T8H10 was added at three concentrations (Log3, 5 and 7 cells ml^{-1}) in 0.22µm filtered seawater; a second set of assays used 1 µm filtered seawater. Each well was replicated 6 times, and larvae challenged for 2 d.

For all bioassays, larvae in each challenge well were sampled every 24 h for the period of the assay up to 3 d (see Table 5-1 for challenge duration). Duplicate larval samples were collected for mortality estimation using a sterile disposable 2 ml pipette, and examined at 400 × magnification using a compound microscope (Leica DM750). Mortality was estimated from a minimum of 50 larvae. Larvae exhibiting disintegration or absence of shell content, or immotile larvae with no ciliary activity or internal organ movement were considered dead. All larvae (dead inclusive) were returned to challenge wells after counting.

Lethal concentration causing 50 % mortality (LC_{50}) was calculated using the method of Reed and Muench (1938).

$\text{Log } LC_{50} = \alpha * \text{Log}(b) + c$

Where α is the proportional distance of 50 % end point = (50 %-mortality at dilution next below)/ (mortality next above- mortality next below); b= dilution rate of bioassay used in this experiment is by 100 fold; c = the log of concentration (cells ml^{-1}) where mortality is below 50 %.

A LC_{50} of greater than 10^8 cells ml^{-1} was considered avirulent, whereas a LC_{50} of 10^4 to 10^5 cells ml^{-1} was considered virulent (Mittal et al., 1980).

5.3.3 16S rRNA gene sequencing

All 10 isolates (T8H1 to T8H10) were identified by 16S rRNA gene sequencing using universal primers 27F and 1492R (Weisburg et al., 1991). After 48 h incubation at 25 °C, single bacterial colonies were picked using a sterile pipette tip

and resuspended in 500 µl of sterile double distilled water in a sterile 1.5 ml Eppendorf tube. The colony was dispersed by vortex mixing for 2 min, and then stored at -20 °C until defrosted and used directly as template in PCR.

PCRs were performed in a C1000 Touch^{TM} Thermal Cycler (Bio-Rad, USA). Final reaction volumes of 25 µl consisted of 12.5 µl 2 × Red Immomix PCR super mix at manufacturers standard concentration (Bioline, USA), 2 µl of colony suspension as DNA template, 0.4 µM concentration of each primer. The thermocycling program consisted of an initial 10 min denaturation at 96 °C, followed by 35 cycles of 95 °C for 1 min, 49 °C for 1 min and 72 °C for 2 min; followed by a final extension step of 10 min at 72 °C. PCR amplicons were electrophoresed through 1% (wt/vol) agarose gels, stained with GelRed (Biotium, USA) and visualised under UV light. PCR products of expected size (circa 1,500 bp) were purified using a MoBio PCR CleanUp Kit (MoBio, USA) as per the manufacturer's instructions. The purified amplicons were sequenced in both directions using Big-dye terminator sequencing (Applied Biosystems, USA) with the forward and reverse amplification primers. Sequence reaction electrophoresis was carried out by the Ramaciotti Centre for Genomics, University of New South Wales (Sydney, Australia). Raw sequence electropherograms were viewed and corrected manually using Geneious v5.4.6 software (Biomatters, New Zealand). Consensus sequences of each strain were determined by pairwise alignmemt of forward and reverse sequences obtained from each strain. The assembled strain sequences were used as queries for sequence matching using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/) to establish preliminary identity and nearest neighbour taxa of isolates. Near complete 16S rRNA sequence (1.4kb) of all available 39 type strains of *Pseudoalteromonas* were

acquired from a public access website (http://www.bacterio.net/*Pseudoalteromonas*.html) and aligned using Geneious Alignment tool with default settings, as implemented in Geneious Pro version 5.4.7. A phylogenetic tree was constructed from Tamura-Nei distances using the Neighbour-Joining (NJ) algorithm implemented in Geneious Support for branching topology and clustering was determined via the bootstrap approach using 1000 bootstrap replicates). The analysis was rooted using *Alteromonas macleodii* DSM6062^T, *Vibrio cholerae* CECT514^T, *Shewanella putrefaciens* LMG26268^T, *Pseudomonas aeruginosa* DSM50071^T as outgroups.

5.3.4 Proteolytic activity

Proteolytic activity of representative isolate T8H10 was examined using double layer marine agar-milk agar according to the methods of Sizemore and Stevenso (1970). After 48 h incubation at 25 °C, transparent zones around colonies in the milk agar layer are indicative of proteolytic activity (degradation of the casein). Proteolytic activity of extracellular products (ECPs) was determined using an azocasein assay (Sigma Chemical Co., St Louis, MO) as described by Teo et al. (2003). Briefly, isolates were grown in Marine Broth (MB, Difco) at 25 °C for 48 h on a shaker to stationary phase as determined from plate counts. The supernatant was harvested by centrifugation (3200 × *g*, 22 °C, 10 min) and 250 µl added to 250 µl azocasein (5 mg ml^{-1}) prepared in 50 mM Tris-HCL buffer of pH 8.0. The mixture was incubated at 37 °C for 2 h and the reaction stopped by addition of 500 µl cold 10 % trichloroacetic acid. The mixture was then centrifuged at 12000 × *g* at 4 °C for 5 min, and 500 µl of the supernatant mixed with 500 µl 1M NaOH. Absorbance was

measured at 440 nm using a Nanodrop 8000 spectrophotometer (Thermo Scientific, USA). One unit of protease activity was defined as the amount of enzyme that produces an increase in absorbance of 0.01 after 2 h incubation at 37 °C. Virulent *V. tubiashii* strain 09/2885-1 was included as a protease positive control (Binesse et al., 2008; Hasegawa et al., 2008).

5.3.5 Histopathology

Larval challenges with with Log 7 cells ml^{-1} were repeated using fresh -80 °C cryopreserved isolate T8H10 to produce larvae for direct microscope examination and histopathology. Mussel larvae at 7 dpf, were sourced from a commercial hatchery and maintained at density 20 larvae ml^{-1} in a 300 ml beaker in 0.22 µm filtered seawater with gentle aeration. Larvae were sub-sampled at the beginning of the experiment and every 24 h for 4 d. Larvae were concentrated by centrifugation at 1,000 × g for 45 s, transferred into a 1.5 ml Eppendorf tube and fixed in Davidson's Fixative (Shaw and Battle, 1957) at 4 °C for 72 h. The fixative was pipette removed (larvae settled at bottom tube) and larvae rinsed twice with distilled water, preserved and transported in 70 % ethanol. Histological preparation was carried out by Diagnostic Services, Animal Health Laboratory of Department of Primary Industries, Water and Environment (DPIWE) (Launceston, Tasmania). Larvae were pelleted at 500 × *g* for 2 min and 70 % ethanol removed by pipette. Then, 1 ml of molten agar (Amyl Media, RM250) was pipetted into the tube on top of the larvae and the 1.5 ml Eppendorf tube in the freezer for 5-10 min. The solidifed agar was removed from the tube, cross sectioned and the sections containing larvae then placed into a histology cassette and processed through a 15 h overnight procedure in the TP1050 tissue

processor (Leica Microsystems, Wetzlar, Germany). Paraffin embedded agar was orientated on the EG1160 (Leica), embedded in paraffin wax (Surgipath Paraplast, 39601006, Leica), sectioned at 3 µm using Microm HM340E (Microm International GmbH, Walldorf, Germany) and adhered to microscope slides (Menzel Glaser, Braunschweig, Germany) for 20 min at 60 °C. Sections were deparaffinised, rehydrated and stained using Jung autostainer XL (Leica) for Haematoxylin (Harris' Haematoxylin, AHHNA, Australian Biostain) and Eosin, dehydrated cleared and mounted in CV Mount (Leica, 046430011).

5.3.6 Statistical analysis

Percentage mortality data were arc-sine square root transformed for normality and subjected to one-way and two-way ANOVA (α=0.05) implemented in SAS Enterprise statistical software v5.1 (SAS Institute Inc., Cary, NC, USA). Post-hoc pairwise comparison was carried out using Tukey's post-hoc test.

5.4 Results

5.4.1 Seawater and larval bacterial plate counts

The seawater and larval samples of a larval culture suffering high level of morbidity (>90 % of immotility) contained $2.4 \times 10^5 \pm 0.58 \times 10^5$ CFU ml^{-1} seawater and $9.4 \times 10^2 \pm 5.85 \times 10^2$ CFU $larva^{-1}$. However, TCBS agar did not detect any colonies.

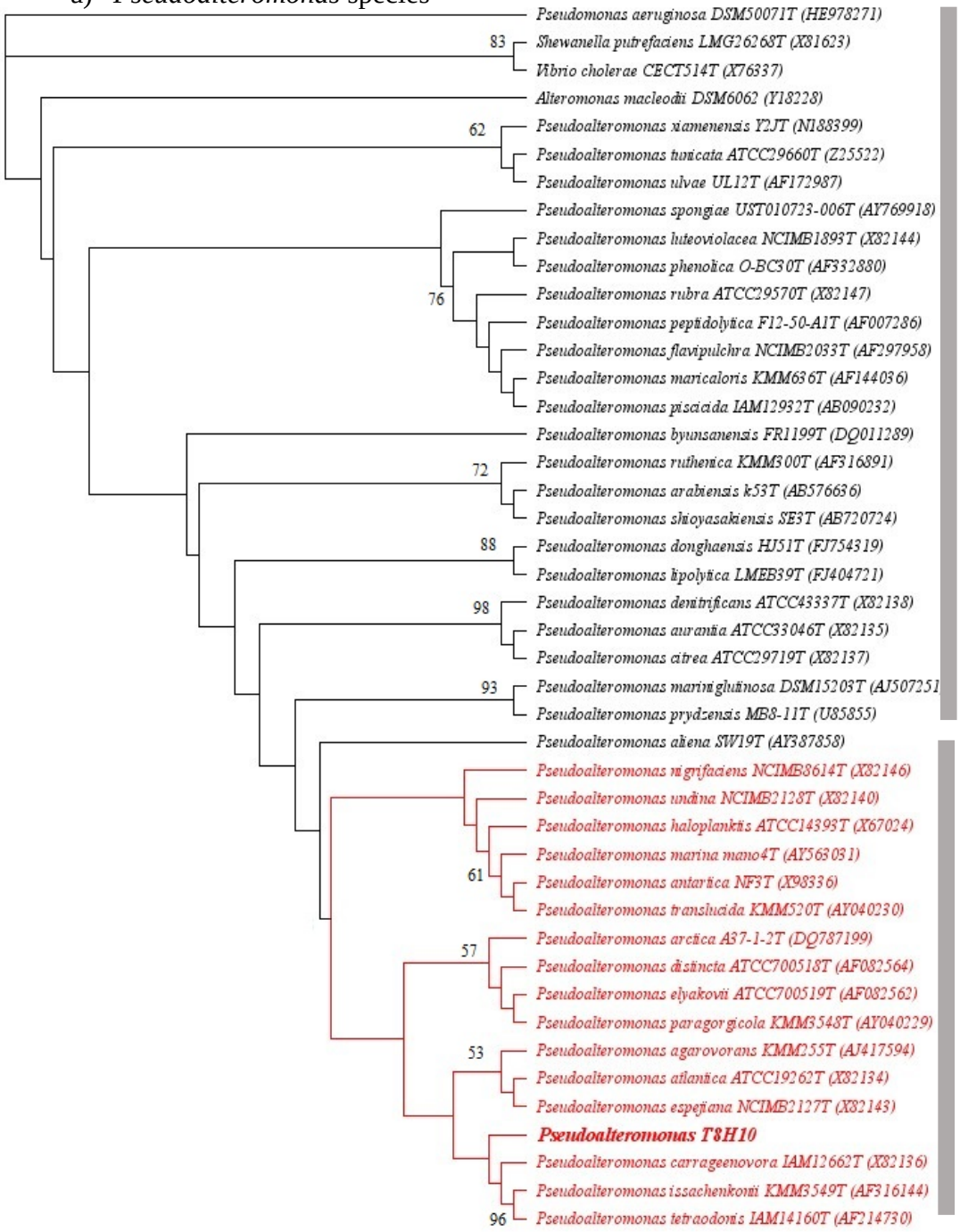
5.4.2 DNA sequencing

The targeted 1.4 kbp 16S rRNA amplicon was successfully amplified and sequenced from all 10 bacterial isolates. After sequence correction, trimming of poor-quality

data, and comparative alignment of forward and reverse sequence, the unambiguous corrected sequence recovered ranged from 1196 to 1388 bp. All 10 isolates were identical over 1196 bp sequence, therefore the sequence of the longest isolate T8H10 was used in two phylogenetic analyses. The first analysis (Figure 5-1a) involved alignment and comparison with 39 published 16S rRNA sequences of *Pseudoalteromonas* type strains resulting in an alignment dataset comprising 1388 nucleotide positions.

Isolate T8H10 clustered within the genus *Pseudoalteromonas* in a distinct sub-group that harbours non-pigmented species (Figure 5-1a). The second analysis of the non-pigmented *Pseudoalteromonas* cluster using 14 type and 30 reference strains showed that isolate T8H10 clusters within a group of strains closely related to *Pseudoalteromonas carrageenovora* (Figure 5-1b) at similarity of 99.1%.

a) *Pseudoalteromonas* species



b) Non-pigmented *Pseudoalteromonas* strains

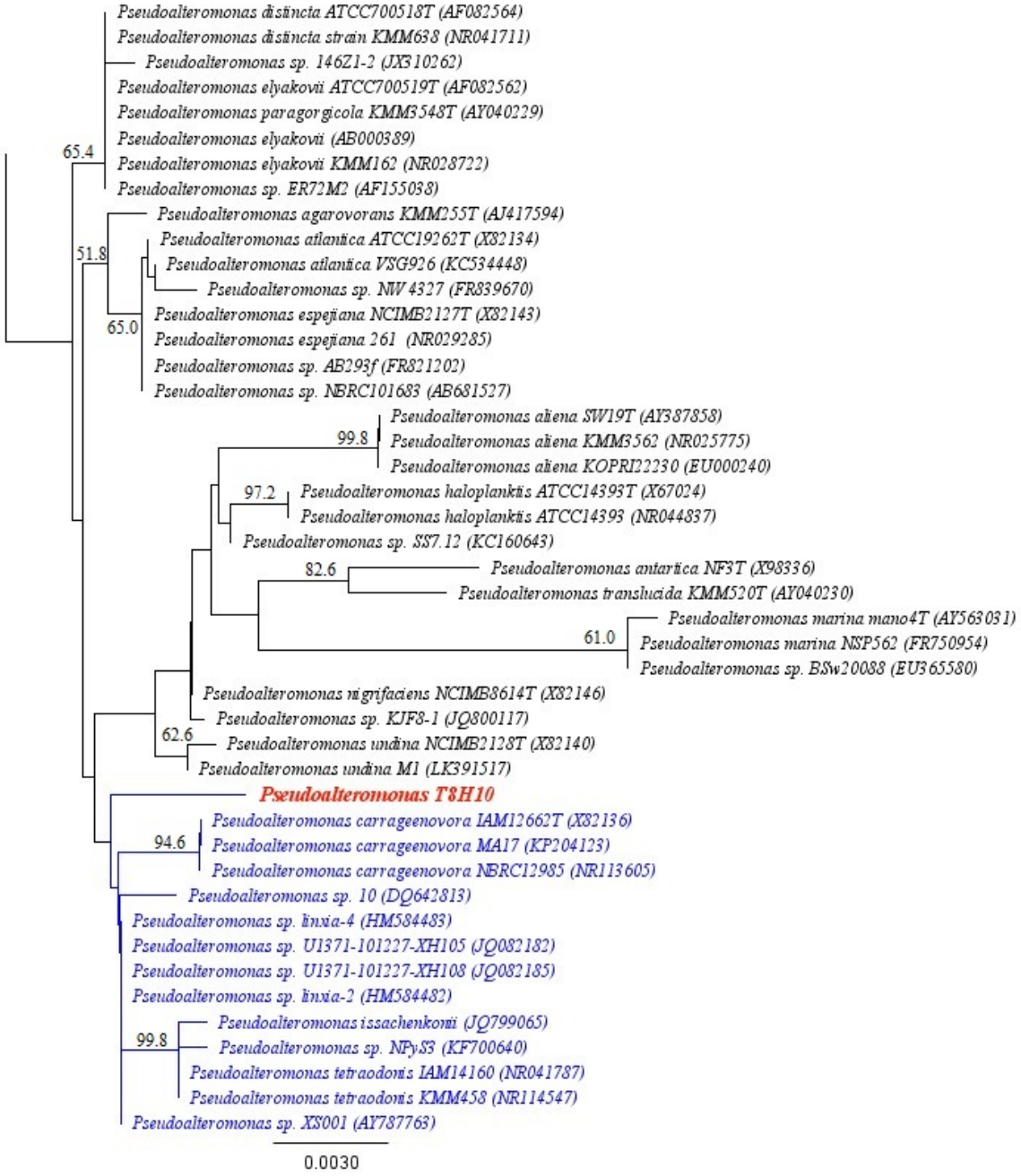


Figure 5-1 Phylogenetic position of isolate T8H10 within a) *Pseudoalteromonas* species and b) sub-cluster of non-pigmented *Pseudoalteromonas* strains based on 16S rRNA gene sequences (1388 bp in length). Tree constructed from Tamurai-Nei distances using the Neighbour-joining method. Branch points with >50% bootstrap (1000 replicates) are shown. Accession numbers for all type and reference sequences are provided in brackets.

5.4.3 Larval challenge bioassay

In the 1 μm filtered seawater, *Pseudoalteromonas* T8H10 produced higher mortality of 2 dpf mussel larvae than the negative controls at a Log 7 cell concentration (Figure 5-2). Mean mortality reached 55.1±3 % in the first 2 d of challenge and plateaued at 3 dpc onward, attaining a total mortality of 66.5±3 % at 4 dpc. These were significantly lower than that of positive controls using virulent the *V. tubiashii* (100 % mortality at 2 dpc).

Similar bioassay challenges using 22 dpf larvae also observed comparable 52 % mortality at 2 dpc. However, mortality did not plateau but increased to 84 % at 3 dpc which coincided with an unintentional increase of incubation temperature from 22.6 °C to 24.2 °C (Figure 5-3).

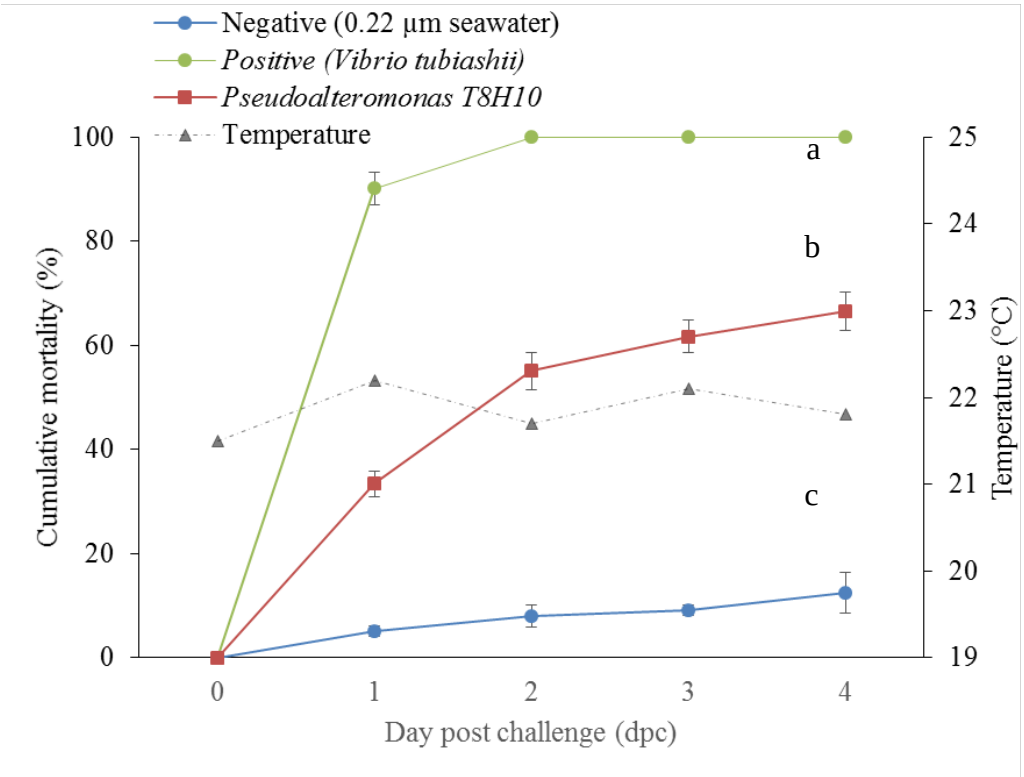


Figure 5-2 Cumulative mortality (± SE %) of 2 dpf mussel larvae and diurnal variation of temperature in the presence of Log 7 cell ml^{-1} of the representative *Pseudoalteromonas* isolate T8H10. Each challenge was carried out in triplicate wells. Negative control received 0.22 µm filtered seawater, positive control used Log 7 cell ml^{-1} *Vibrio tubiashii* (Isolate ID: 09/2885-1). Different letter indicates significant difference in mortality at 4 dpc (one-way ANOVA F=104.5, df 2, p<0.0001)

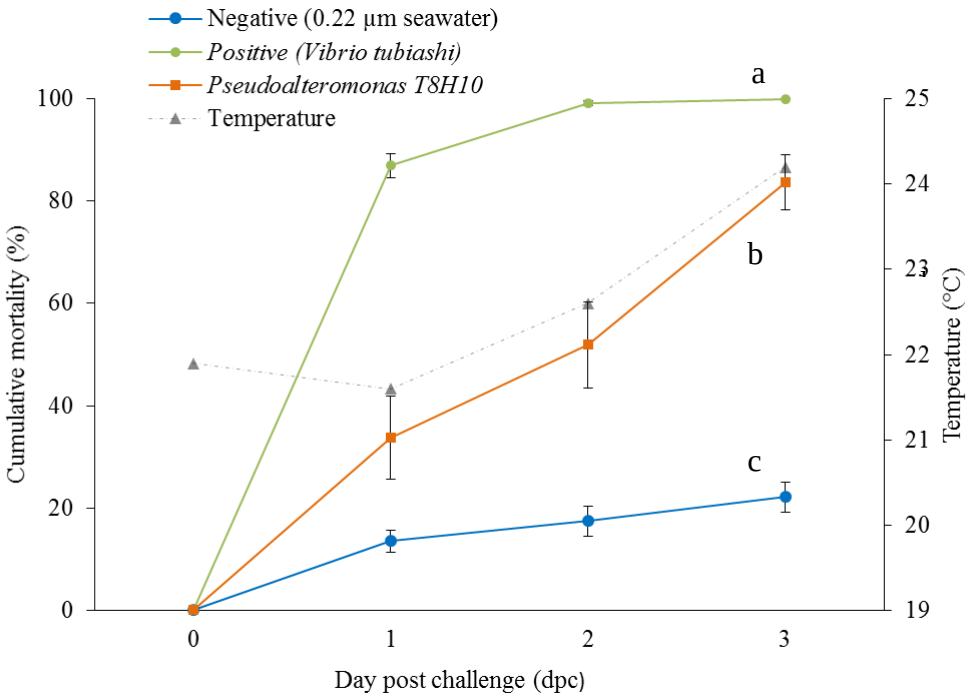


Figure 5-3 Cumulative mortality (± SE %) of 22 dpf mussel larvae in the presence of initial concentration of Log 7 cells ml^{-1} of the representative *Pseudoalteromonas* isolate T8H10 in 1.0 µm filtered seawater. Negative controls received 0.22 µm filtered seawater, positive control used Log 7 cells ml^{-1} *Vibrio tubiashii* (Isolate ID: 09/2885-1). Each challenge condition was tested in 6 replicates using 12 well plate, each containing 125-150 larva in 5 ml seawater. Incubator temperature rose unintentionally from commercial standard 22.6 °C at 2 dpc to 24.2 °C at 3 dpc due to unusually warm weather. Different letters indicate significantly different mortality at 3 dpc (one-way ANOVA F=75.92, df 2, *p*<0.0001).

Isolates freshly revived from cryopreserved stocks showed increasing cumulative mortality (sterile seawater F=130.45, df 1, $p<0.0001$; non-sterile seawater F=205.04, df 1, $p<0.0001$) with increasing challenge concentration whilst repeated subculturing of isolate T8H10 resulted in mortality not significantly higher than sterile seawater controls in both sterile and non-sterile seawater bioassay (Figure 5-4), even at Log 7 cells ml^{-1} (sterile seawater F: 0.94, df 3, 20, $p=0.4380$; non-sterile F: 0.82, df 3,

20, p=0.4998). Freshly cultured T8H10 from -80 °C stored cells not at Log 3 produced significantly higher larval mortality than controls in both 0.22 µm and 1 µm filtered water Log 5 and Log 7 cells ml^{-1}. At 2 dpc, the LC_{50} of T8H10 to 6 dpf mussel larvae was higher in non-sterile seawater (at 2.2 ± 1.6 × 10^6 cells ml $^{-1}$) compared with sterile seawater (at LD_{50} 3.3 ± 2.1 × 10^4 cells ml $^{-1}$) (one-way ANOVA F=21.7, df 1, p=0.0009).

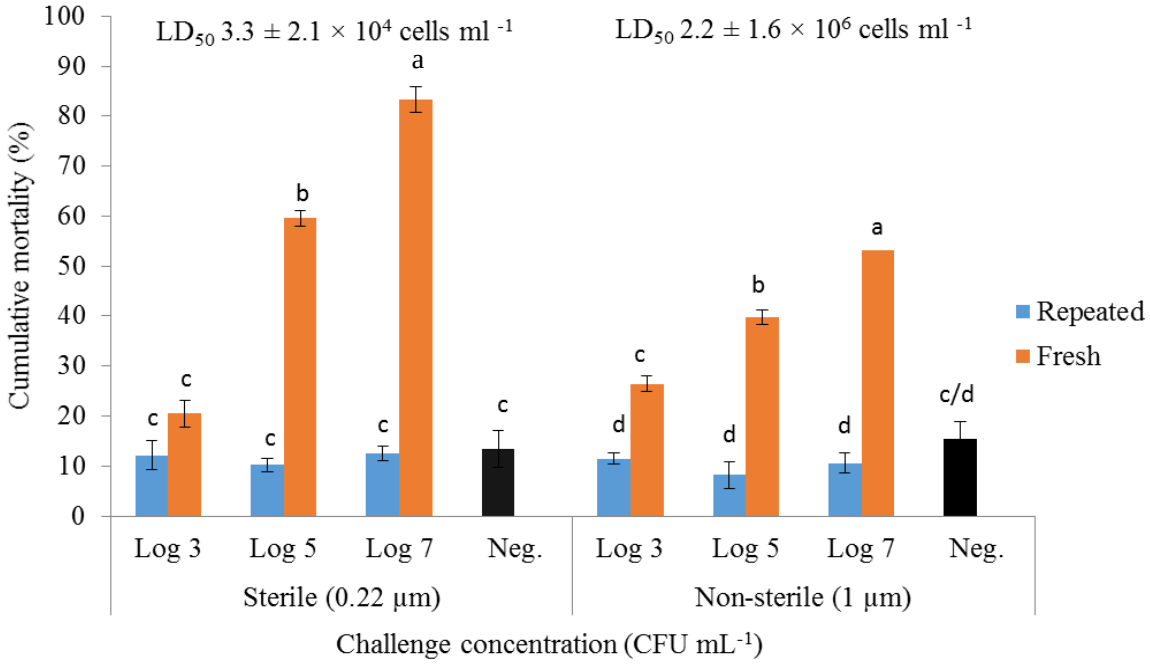


Figure 5-4 Cumulative mortality (± SE %) of 6 dpf mussel larvae over a 2 day duration challenged with representative isolate T8H10 sourced from repetitively subcultures and fresh cryopreserved stock. A series of challenge concentrations (Log 3, 5 and 7 cells ml^{-1}, negative control (Neg.) were examined in 0.22 µm filtered sterile and 1.0 µm filtered non-sterile seawater. Each challenge condition was performed in 6 replicates using 12 well plates, each containing 125-150 larva in 5 ml seawater. Within each seawater, different letters denotes significantly different mortality(sterile, one-way ANOVA F=44.67, df 7 40, p<0.001; non-sterile F=53.46, df 7 40, p<0.0001). LC_{50} for each seawater condition is also presented.

5.4.4 Behaviour, morphology and symptoms of *Pseudoalteromonas* T8H10 infected larvae.

Mussel larvae challenged by *Pseudoalteromonas* isolate T8H10 was characterised by abnormal velum activity and degradation, which progressed through 5 stages over a 3 d period. The visible stages, symptoms and progression of the disease are summarised in Table 5-2. Irregular swimming behaviour of mussel larvae was observed during the first 12 h since inoculation of the bacteria. Early stage challenged larvae exhibited erratic swimming behaviour with bursts of rapid swimming interspersed with immotility and irregular retraction of vela. The pause in swimming coincided with high velar activity (repetitive retraction and protraction). By 24 h, the erratic swimming resulting in limited ability to maintain position in the culture and larvae were observed to aggregate at the bottom container presenting a dense cluster of larvae. At 400 × magnification, larvae showed abnormal protrusion of the vela, which was often deformed and paralysed (severe stage, Figure 5-5b and c) and unable to retract the vela when the shell was closed. The exposed vela became abnormally clumped (Figure 5-5c) resulting in larvae with erratic spinning behaviour likely due to impaired swimming capacity. Between 32 to 48 h after challenge, the vela was further reduced and detached from the larvae (completion stage, Figure 5-5d). In the culture container, detached vela were observed in the water column and larvae became immotile. By 48 h, mortality was prevalent in cultures (completion stage). Bacterial swarming around larvae was only observed in a small proportion of larvae during velar deformation and again at the completion stage where many larvae where immotile and terminally diseased. After 48 h, post-mortem degradation of

larvae increased rapidly resulting in empty shells and increasing ciliate abundance feeding on dead larval tissue (Figure 5-5f).

Table 5-2 Categorisation of disease development in 7 dpf mussel larvae challenged with concentration Log 7 cells ml^{-1} of *Pseudoalteromonas* isolate T8H10 based on wet mount (400 ×) and histopathology examinations (H and E stain, 400 × and 1000 ×) at 12, 24, 32, 48 and 60 h post challenge in 0.22 µm filtered seawater.

Stage	Period (h)	Gross observation	Histology
Early	0─12	Short rapid burst of swim with intermittent and repetitive retraction of the vela	No visible abnormal tissue, compact shell content and intact vela in retracted position (Fig. 6a, 6b)
Mid	12─24	Paralysed vela, reduced motility with erratic spinning, larval aggregation at bottom of culture vessel (Fig. 5b)	Mild protrusion of vela, Shell valves closed but vela not retracted, necrosis of intestine (Fig. 6c, 6d)
Severe	24─32	Gross protrusion and deformation of the vela (clumping), larvae sedentary but with visible intestinal movement (Fig. 5c, 5d)	Necrosis of velar tissue, vela ciliated, digestive gland and intestines reduced in complexity, no evidence of bacterial attachment (Fig. 6e, 6f)
Completion	32-48	High proportion of dead larvae(no intestinal activity), vela reduced significantly, detached vela could be observed in seawater, bacterial swarming very rare (Fig. 5e)	Velar necrosis, de-ciliation and detachment, intestines significantly reduced in complexity resulting in abnormally voided shell valves. Stomach and style sac intact but "balloon" progressively in the absence of intestines, no evidence of bacterial invasion of tissue(Fig. 6g, 6h)
Post mortem	48─60	Empty shells, ciliates and protozoans attack remaining shell contents (Fig. 5f)	Shells lack content, remnant of style sac and stomach persist (Fig. 6h)

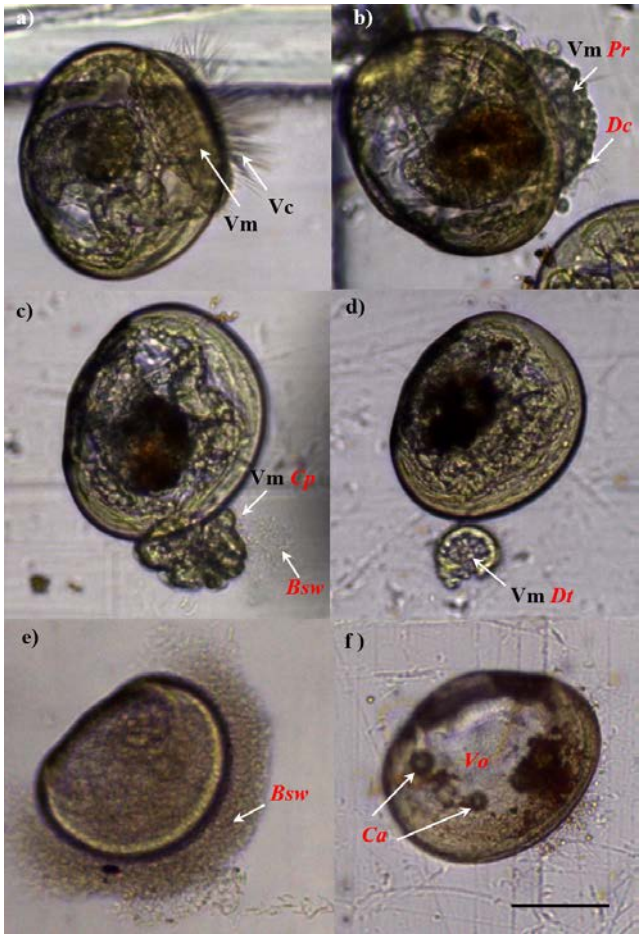


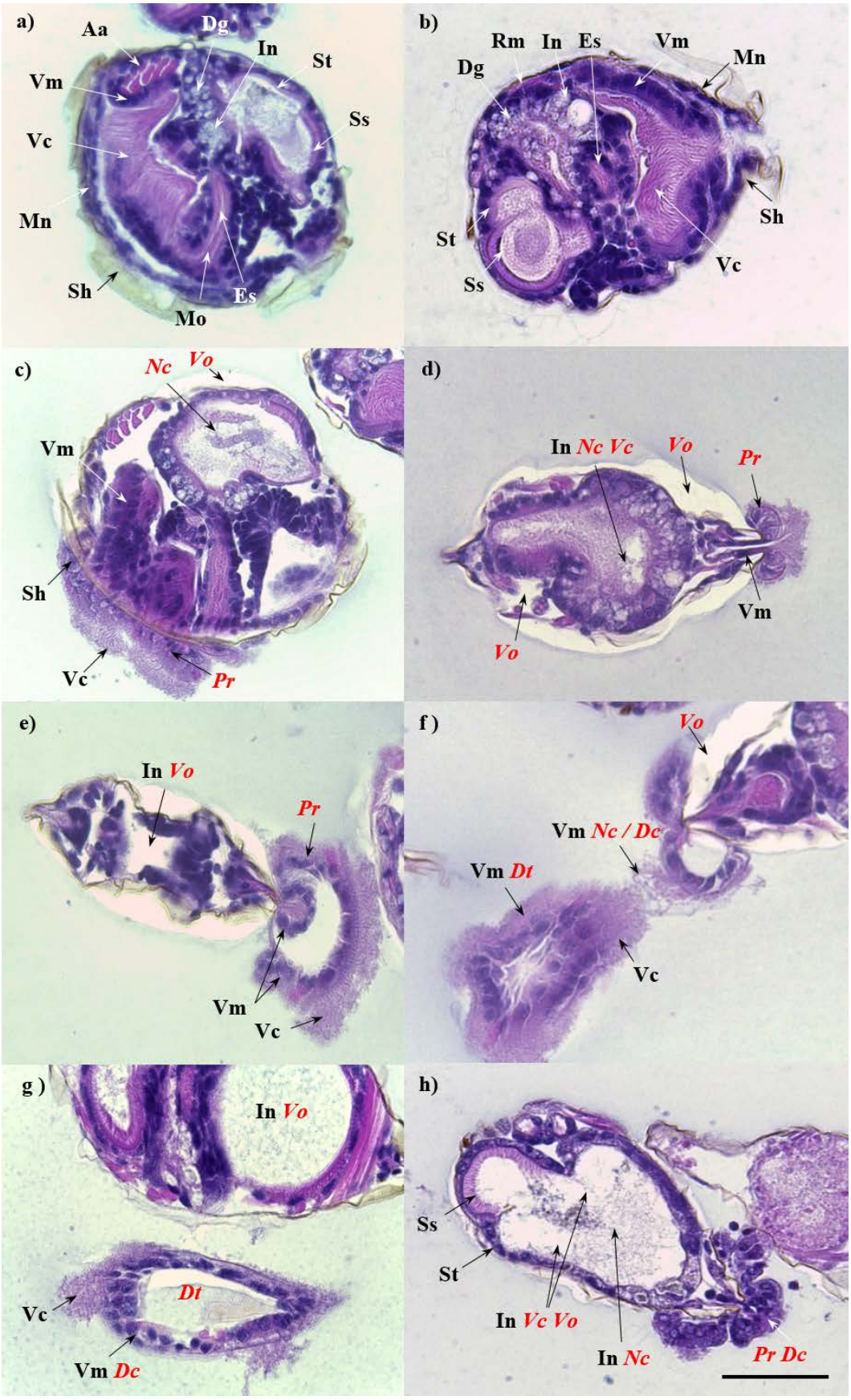
Figure 5-5 Wet mount observations of a) 7 dpf mussel larvae prior to challenge of *Pseudoalteromonas* isolate T8H10 showing normal shape and protraction level of velum (Vm, at sedentary position when viewed), straight and dense Velar cilia (Vc); infected larvae at b) 12 h showing early protrusion (*Pr*), rough surface and deciliation (*Dc*) of velum; c) 24 to 32 h showing further protrusion, complete deciliation, clumping (*Cp*) and bacterial swarming (*Bsw*) around the velum (Vm), d) 32 to 48 h showing progressively reduced and detachment (*Dt*) of velum, e) 48 h showing bacterial swarming (*Bsw*) around larvae and sloughing of bacterial cells; and f) 48-72 h showing void spaces in shell contents with ciliate attacks (*Ca*) of remaining shell contents. Bar, 75 µm.

5.4.5 Histology

From examination of larvae before and after fixation, contact of both healthy and living larvae with Davidson's fixative causes larvae to retract the vela and close valves (Figure 5-6a and b). However, at all infection stage (early through severe stage, see Figure 5-6a to 5h) larvae presented closed valves with an unretracted

velum. The protraction is supported by a thin stalk of velum retractor muscle (Figure 5-6d). The level of protrusion changed with increasing stage (and severity) of disease, with early stage showing partial and severe stage gross protrusion and detachment of velum. The point of detachment could be identified by necrosis of velar tissue, as indicated by remnants of velar cells (dark nucleus, distinctively columnar shaped and ciliated) remaining intact after larvae had lost the vela (Figure 5-6f). The severe stage of infection displayed deciliation of the detached velar tissue. The gross protrusion of the vela resulted in an abnormal void within the shell which could be easily differentiated from unchallenged larvae (compare Figure 5-6b and e).

Necrosis of the gut, indicated by loss of ciliated epithelial cells (Figure 5-6c and d) was observable as early as 12-24 h post challenge but was not prevalent until 48-60 h post challenge when mass mortality has occurred (Figure 5-6h). The intestines and digestive glands at this stage were significantly reduced due to necrosis and vacuolation of cells leading to increasing voided spaces in individuals. Attachment and invasion of bacteria was not evident in fixed/prepared specimens even though bacterial swarming was observed to surround dying larvae in wet mount preparations.


a)
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h)
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In Nc
Pr Dc

Figure 5-6 Hematoxylin and Eosin (H and E) histology examination of a) sagittal to oblique section and b) transverse section of a 7 dpf mussel larvae prior to challenge of *Pseudoalteromonas* isolate T8H10 showing shell (Sh), anterior abductor muscle (Aa), mantle (Mn), velum (Vm), velar cilia (Vc), mouth (Mo), esophagus (Es), stomach (St), style sac (Ss) , digestive gland (Dg) and intenstine (In). Gill and posterior abductor muscle is not visible. Unchallenged larvae showed velum in a normal retracted position, has compact and well organised organs. Challenged larvae at c and d) 12 h showing early protrusion (*Pr*) of velum with shell closed, and void spaces (*Vo*) as a result of protrusion, shedding of cilia from necrosis in stomach/style sac compartment; d) early necrosis (*Nc*) and vacuolation (*Vc*) of intestines (In); e) 24-32 h showing progressive velum protrusion (*Pr*) and the resultant void spaces, velum still ciliated and attached by a thin stalk of velar retractor muscle; f and g) 32-48 h showing necrosis (*Nc*) of velar attachment muscle, deciliation (*Dc*), detaching and completely detached (*Dt*) velum; h) 48-60 h showing dead larvae with widespread necrosis (*Nc*), vacuolation (*Vc*) of intestines and notably void shell content. Bar, 75 µm.

5.4.6 Protease activity

Pseudoalteromonas isolate T8H10 exhibited limited proteolytic activity when grown on marine-milk agar with clearing of agar restricted to near the boundary of colonies (Figure 5-7a). In contrast, proteolytic activity of the comparative control, *V. tubiashii*, was strong and highly diffusive, resulting in wide zones of casein hydrolysis. Using the azocasein assay, protease activity (U ml^{-1}) of extracellular products (ECPs) of isolate T8H10 was 3.12 ± 0.45 U ml^{-1}), 20 times less than the activity of *V. tubiashii*. (59.67 ± 0.24 U ml^{-1}).

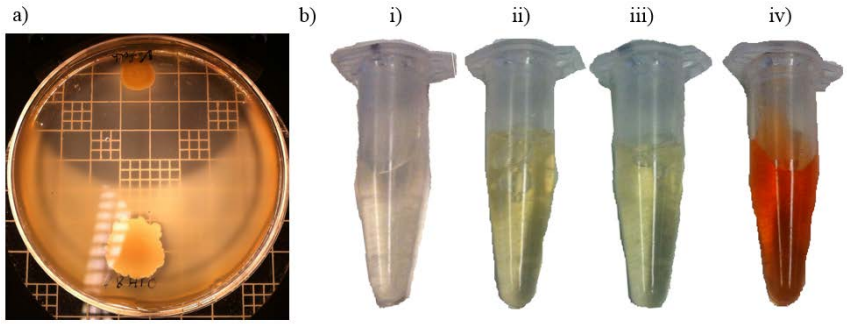


Figure 5-7 a) Marine-milk agar incubated for 48 h at 25 °C showing limited clearing of casein protein by *Pseudoalteromonas* isolate T8H10 (bottom colony) and extensively cleared zone (radius of 4 cm) by *Vibrio tubiashii* Isolate ID: 09/2885-1 (top colony). Casein hydrolysis activity of bacterial extracellular products (ECPs, collected after 48 h culture in MB at 25 °C) were examined for i) blank (uninoculated MB); ii) *Pseudoalteromonas* isolate T8H8; iii) *Pseudoalteromonas* isolate T8H10; and iv) *Vibrio tubiashii* Isolate ID: 09/2885-1. Intensity of azo dye (orange color measurable at 440 nm) is proportional to proteolytic activity.

5.5 Discussion

The current prevailing view of *Vibrio* as a major cause of bacillary necrosis is largely driven by the high number of studies demonstrating lethality of *Vibrio* isolated from diseased larvae. This is facilitated by their relatively easier recovery on selective media making them a convenient study subject in larval challenge bioassays. However, importance of *Vibrio* may have been overstated as our studies in Chapter 2 demonstrated *Vibrio* outbreaks during times of disease can also be an opportunistic growth response to the already diseased larvae therefore contaminating the scene of disease. Furthermore, culturable independent data in Chapter 3 and 4 showed *Vibrio* generally comprises less than 1.5 % of total bacterial communities in moribund larval culture suggesting the possibility of overlooking other bacteria in the role of disease.

The role of *Pseudoalteromonas* in larval disease was suspected in Chapter 4 due to its overwhelming dominance of up to 70 % of total bacterial community in diseased cultures. However, its comparable dominance in healthy larval culture complicates the association with disease. Coincidentally during a separate mass mortality event of laboratory larval rearing, this study isolated and showed for the first time the opportunistic potential of a strain of *Pseudoalteromonas* in mussel larvae cultures. The fact that all 10 randomly isolated colonies were identical indicates this bacterial strain occurred in high concentration in the larval culture suffering mass mortality. The capability of *Pseudoalteromonas* to dominate mussel larval culture bacterial community and harbour pathogenic bacterial strain warrants renewed attention of their ecological and disease roles in larval cultures.

Currently including 39 species, the bacterial genus *Pseudoalteromonas* belongs to class *Gammaproteobacteria*, and has great marine ecological significance particularly through production of bioactive compounds (Bowman, 2007). *Pseudoalteromonas* are well studied because of their beneficial potential in producing algicidal, anti-fouling, antimicrobial, antifungal and various pharmaceutical compounds. This finding of a *Pseudoalteromonas* strain capable of lethal interaction with mussel larvae is not totally unexpected as various strains of this bacterial species produce poor survival in diverse marine organisms ranging from reef sponges, *Rhopaloeides odorabile* (Choudhury et al., 2014), farmed crab, *Cancer pagurus* (Costa-Ramos and Rowley, 2004), sea cucumber, *Apostichopus japonicus* (Liu et al., 2010a), farmed gilfhead sea bream, *Sparus aurata L.* and European sea bass, *Dicentrarchus labrax L.* damselfish (family *Pomacentridae*) (Nelson and Ghiorse, 1999) to even marine macroalgae, *Gracilaria gracilis*

(Schroeder et al., 2003) and *Laminaria* seaweed (Narita et al., 2003). The only study so far that has reported involvement of *Pseudoalteromonas* in bivalve larval stock mortality is in scallop hatcheries (Torkildsen et al., 2005), indicating the lack of information in disease role of *Pseudoalteromonas* in bivalve larvae.

Shedding of the velum is normal in metamorphosing larvae preparing for transition to life attached on substrate (Elston, 1999). However, challenged larvae for histology examination (i.e Bioassay 4 of Table 5-1) were approximately 2 weeks from settlement and had not yet developed typical settlement behaviour such as extension of foot in search for settlement substrate. Therefore, *Pseudoalteromonas* T8H10 can cause premature and abnormal velar detachment. Notably, no bacterial attachment or invasion were observed in the deformed vela prepared on histology slides though wet mount observations only in very few occasion observed swarming of bacteria in affected larvae. This indicates direct bacterial invasion is unlikely a mechanism of pathogenicity of isolate T8H10. Tissue necrosis without presence of bacterial attachment are rare but have been previously noted in oyster larvae challenged with pathogenic *Vibrio* spp. (Tubiash et al., 1965; Jeffries, 1982). Follow-up works using a more sensitive technique such as the fluorescence in-situ hybridization (FISH) would be necessary to confirm absence of bacterial cells attachment/invasion in the disease process of isolate T8H10.

The velar deformed mussel larvae still showing signs of internal organ activity, however had aggregated at the bottom of culture container due to loss of locomotion. These may have share similarities to a phenomenon called larval aggregative behaviour characterised with larval swarming at bottom of culture tank which almost

certainly precede larval death in commercial cultures (Disalvo et al., 1978; Sugumar et al., 1998b; Prado et al., 2005). However, it is worthy to mention that the loss of swimming activity observed in this study is different to several studies (Birkbeck et al., 1987; Nottage et al., 1989). The aforementioned authors characterised a small molecular weight proteinase toxin produced by marine *Vibrio* that is capable of ceasing ciliary activity of velum. In contrast, high concentration of *Pseudoalteromonas* T8H10 in seawater appears to lead to disintegration of the velar retractor muscle (rather than ceasing of velar cilia) resulting in rapidly deterioration of swimming activity. This study documented for the first time, a strain of *Pseudoalteromonas* in the deformation of hatchery reared bivalve larval vela. The pathological examinations agree with Elston (1999) that such velar deformation is potentially mediated by toxin, and rightly fits Type II pathogenesis of bacillary necrosis (Elston and Leibovitz, 1980). This study characterised for the first time the progression of velar deformities. Briefly, these includes over active retraction of vela which escalates to velar paralysis causing impaired swimming in spinning motion. Necrosis of velar tissue and de-ciliation then sets in resulting in clumping, detachment of vela and ultimately high mortality rate at 48 h.

Whilst the characteristics of larval velar deformation so far has been consistent with activity of extracellular proteinases (Elston and Leibovitz, 1980), this study shows *Pseudoalteromonas* T8H10 possesses a relatively weak proteolytic activity based on the substrate casein. If proteases are involved, they may have a high level of substrate specificity for particular protein/s associated with the velar retractor muscle. It is worthy of mention that Venkateswaran and Dohmoto (2000) demonstrated *Pseudoalteromonas peptidolytica* produces specific proteases that

are capable of cleaving mussel thread protein complex, which otherwise are not degradable by a range of marine bacteria. Given the example of mussel thread degradation and the fact that non-pigmented species of this genus has been reported to be active in production of secondary metabolites (Bowman, 2007), additional research with different substrates are necessary to examine the lysis activity of extracellular products of *Pseudoalteromonas* T8H10. Alternatively, the observations of weak proteolytic activities based on the azocasein assay and marine-milk agar could be due to absence of host factors. This is not impossible because Dubert et al. (2016) using green fluorescent tagged protein (GFP) shows that several species of *Vibrio* pathogenic to bivalves colonise and proliferate rapidly when the bacterial cells are in contact with the digestive system specifically digestive gland and intestines, leading to authors to believe host's chemical/surface cues in triggering virulence factors.

Bioassays data show *Pseudoalteromonas* strain T8H10 can cause 50 to 60 % mortality in D stage (2 dpf), late umbone (7 dpf) and pediveliger (22 dpf) larvae when bacterial cell concentration reaches over 10^6 cells ml $^{-1}$ in rearing seawater. This concentration level is considered relatively high compared with typical total viable cells (TVC) readings ranging from 10^3 to 10^5 CFU ml $^{-1}$ in commercial hatcheries (Nicolas et al., 1996; Sugumar et al., 1998b; Magnesen et al., 2013). The disease risk caused by the *Pseudoalteromonas* strain T8H10 in typical larval culture production is likely to be low because of the higher lethal cell concentration (i.e LD_{50} $2.2 \pm 1.6 \times 10^6$ cells ml $^{-1}$ in 1 µm filtered seawater) compared with the established well known virulent pathogens such as *Vibrio tubiashii*, (Elston et al., 2008b; Richards et al., 2015). Good husbandry practices should maintain concentration

below 10^5 cells ml $^{-1}$ (Magnesen et al., 2013) therefore minimising risk of mortality caused by this strain. Furthermore, high bacterial cell concentration of this strain even at 10^7 cells ml $^{-1}$ does not always result in lethality as our study observed significantly reduced virulence associated with repetitive sub-culture, indicating virulence of this *Pseudoalteromonas* strain is modulated by the culture environment (possibly absence of host) and may become silenced under prolonged culture in an artificial environment. Similarly, several studies examining virulence of *Pseudoalteromonas* strain LT-3 isolated from disease scallop larvae also revealed remarkably different lethality ranging from mortality levels not different to un-inoculated controls (Sandlund et al., 2006) to levels comparable to those challenged with scallop pathogen *Vibrio pectinicida* (Torkildsen et al., 2005). These together suggest opportunistic nature of *Pseudoalteromonas* in bivalve larval cultures.

Regulation of virulence in our T8H10 strain was not examined but it is suspected based on published information, the fast growth rate in nutrient rich media employed in this study might have also contributed to our bacteria becoming avirulent. Virulence factors such as surface attachment and production of toxin in pathogenic bacteria are greatly modulated by growth environment, specifically nutrient deprivation (Brown and Williams, 1985). Ombaka et al. (1983) presented data showing artificial media nutrient depletion (most notable for carbon, phosphorus, nitrogen and iron) to mimic *in-vivo* conditions resulted in slower growth rate of a human pathogen *Pseudomonas aeruginosa* which observed marked increased toxin production. Wiersma et al. (1978) also showed that extracellular protease production of a coastal *Vibrio* strain was stimulated by low iron content and dissolved oxygen in growing media. These studies suggests that suboptimal growth conditions may

trigger the bacteria to switch on different metabolic abilities such as extracellular production to have access to certain forms of nutrients. These changes in bacterial activity necessary for survival however could implicate the host negatively. In this study, the removal of host larvae and repetitive culture of isolate T8H10 in MA (Difco) which contains enriched levels of carbon and nitrogen (from peptone and yeast) and iron (ferric citrate) may have altered the conditions under which such extracellular production activity (virulence) is not required.

In this study, the effect of warmer temperature and/or in combination with longer challenge duration observed increased mortality. It is unsure if this was due to increased susceptibility of larvae, increased bacterial virulence or other non-microbial processes (such as depleting oxygen) as a result of the gradual rise of temperature to 24.2 °C. However, Sanchez-Lazo and Martinez-Pita (2012) recommended that rearing temperature of 24 °C produces higher growth rate and larval survival of *Mytilus galloprovincialis*, suggestive that the maximum temperature attained during the bioassay would likely be within the thermal limit of the larvae. A logical follow-up experiment would be to determine how higher thermal limit of mussel larvae at 24 °C can contribute to increased lethality either through non-microbial processes or factors such as multiplication of T8H10 cells resulting in higher dosage in the bioassay and/or up-regulation of virulence factors.

Using qPCR targeting several virulence genes, Liu et al. (2016) showed concentration of bivalve pathogens (*Pseudomonas* spp. and *Vibrio* spp.) in bivalve farms seawater increases when seawater warms up to 23 °C, suggestive of the potential of T8H10 to rapidly divide resulting in higher cell concentration in bioassay

wells. Up regulation of virulence in T8H10 is also worthy of examination. For example, Kimes et al. (2012) demonstrated multiple virulence factors such as motility, host degradation, antimicrobial resistance and secretion of a tropical coral pathogen *Vibrio coralliilyticus* are not up regulated until seawater temperature attains 27 °C. A follow up temperature dependent series challenge bioassay would provide information to confirm role of temperature in virulence potential of *Pseudoalteromonas* T8H10.

5.6 Conclusion

In conclusion, this study isolated and demonstrated opportunistic potential of a novel strain of *Pseudoalteromonas* to hatchery reared blue mussel larvae. To the best knowledge of this study, this is the first report of *Pseudoalteromonas* capable of causing mortality in mussel larval culture suggestive of the possibility of more undescribed pathogens within this genus. This study establishes that the representative *Pseudoalteromonas* isolate T8H10 shows moderate virulence (LD_{50} 10^6 cells ml^{-1} in 1 µm filtered rearing seawater) but capable of affecting D stage (2 dpf), late umbone (7 dpf) and pediveliger (22 dpf) larvae. This bacterium can disrupt the feeding and swimming organ-velum leading to structural abnormalities and eventually premature detachment from the larvae. However, the link of velar deformities with virulence factor is not established but this study observed variable virulence suggestive of its opportunistic nature. More studies to investigate physiology and virulence of this strain could shed light in better understanding opportunistic nature of bacillary necrosis.

Chapter 6 General discussion: recommendations and further research

6.1 Bacillary necrosis: a chronic problem of bivalve hatchery worldwide

Mollusc aquaculture, which comprises predominantly bivalves, is the biggest marine aquaculture sector worldwide in 2014 producing 16.1 million tonnes, compared with crustacean and fish combined at 13.2 million tonnes (FAO, 2016). The substantial nature of bivalve aquaculture is reflected in the diverse species being cultured. These includes scallop (family *Pectinidae*; *Argopecten irradians*, *Argopecten purpuratus* , *Argopecten ventricosus*, *Euvola ziczac*, *Nodipecten subnudosus*, *Pecten maximus*), clam (family *Veneridae*; *Mercenaria mercenaria*, *Ruditapes decussatus*, *Venerupis philippinarum*), cockle (family *Cardiidae*; *Fulvia mutica*, *Tricadna gigas*), penshell (family *Pinidae*; *Atrina maura*), oyster (family *Ostreidae*; *Crassostrea virginia*, *Crassostrea gigas*, *Crassostrea sikamea*, *Ostrea edulis*), mussel (family *Mytilidae*, *Mytilus galloprovincialis*, *Perna canaliculus*), geoduck (family *Hiatellidae*, *Panope abrupta*). Unfortunately, hatchery larval production of all bivalve species are characterised by sporadic mass mortality caused by bacillary necrosis capable of wiping out whole larval population in a short period of 24 to 48 h (Paillard et al., 2004). Despite over 50 years since the first detailed description of bacillary necrosis, this problem remains difficult to manage. This contributes to bacillary necrosis being the single most serious bacterial problems in bivalve larval rearing worldwide. In Australia, the problem of bacillary necrosis is no less significant with losses

estimated at $500,000 annually, represented an undersupply of spat approaching 50 % of demand (Chapman, 2012). The economic viability of hatchery and the production capacity of grow out is chronically compromised by bacillary necrosis.

6.2 Research outcomes- contribution to the understanding of microbial ecology of bacillary necrosis

The understanding of microbes associated with bivalve larval rearing can help improve current disease risk monitoring, knowledge on how to maintain healthy larvae through better rearing management practices, and provide background information to manage disease. The fact that prophylactic antibiotic reproducibly prevents bacillary necrosis (Nicolas et al., 1996; Eggermont et al., 2014; Holbach et al., 2015) shows larval health is greatly dependent on the microbial quality because of their filter feeding that concentrate bacteria from seawater (Ciacci et al., 2009) and their lack of ability to select for bacteria living external and internally (Beleneva et al., 2003; Preheim et al., 2011; Wendling et al., 2014). The host-bacteria interactions modulated by external conditions play important roles in determining health and vulnerability to bacterial attacks (Schulze et al., 2006; Lemire et al., 2015; Petton et al., 2015).

6.2.1 Association and role of *Vibrio*

Many larval challenge bioassay studies demonstrate pathogenicity of various *Vibrio* strains isolated from diseased larvae resulting in the prevailing view that *Vibrio* are primary pathogens of bacillary necrosis. However, this view is based on limited understanding of *Vibrio*'s dynamic and direct causality in bacillary necrosis. This

thesis using culturable techniques shows that *Vibrio* can be linked with bacillary necrosis, not just at the time of mass mortality (Figure 4-2 of Chapter 4) but in the development of bacillary necrosis (Figure 2-3a and 2-3c in Chapter 2). Chapter 2 shows the occurrence of bacillary necrosis due to overfeeding is associated with a rapid and transient increase in *Vibrio* relative abundance, referred to as *Vibrio* growth spikes which occur in seawater 1-2 d prior to mortality, and in larvae during mortality. This agrees with the earlier study that also described transient *Vibrio* spikes preceding mortality in commercial oyster larval cultures(Chapman, 2012). Given the similar patterns observed in oyster and mussel larval, this indicates that *Vibrio* population changes can be a common characteristic and possibly have roles in the onset of bacillary necrosis.

Mortality caused by bacillary necrosis can proceed rapidly; up to 50 % within 24 h, the typical sampling interval in most scientific studies. This rapid development can confound interpretation of pre- or post-mortem association of *Vibrio*. This is particularly relevant as *Vibrio* species are known to be amongst the fastest growing bacteria (Rehnstam-Holm et al., 2010), allowing them to take advantage of nutrient leaching from dying/dead larvae. The higher concentration in dead/dying larvae allowing easier recovery for various studies including pathogenicity tests and challenge bioassay, perhaps explains the large number of studies focusing on isolation and characterisation of *Vibrio* from bacillary necrosis affected larval cultures.

By employing larval challenge bioassays, this study however did not detect a virulent *Vibrio* that caused mass mortalities in the mussel larval cultures despite *Vibrio*

showing clear changes in abundance in response to overfeeding and larval death. This observation contradicts many studies (eg. Elston et al., 2008b; Kesarcodi-Watson et al., 2009a) because *Vibrio* in this case is unlikely a causative agent therefore warranting care for future studies in prematurely associating *Vibrio* as cause of each of the many bacillary necrosis cases. Assuming that *Vibrio* is causative agent may in fact bias our screening. It is suspected, by selecting higher number of isolates for bioassay challenges, one would potentially find one or few virulent *Vibrio* isolates from any given diverse bacterial community. But, whether these isolates were causative agents of bacillary necrosis cannot be established on the ground of mortality observed using larval challenge bioassay alone because such bioassay does not reproduce natural infectivity.

This thesis indicates that *Vibrio* can have an accessory role in the development of bacillary necrosis. This study shows *Vibrio* that makes up the growth spikes belongs to the *Vibrio splendidus* group, not surprising given their known association with both healthy and diseased bivalve larvae (Prado et al., 2014a) and dominance of *Vibrio* populations in hatcheries (Kwan and Bolch, 2015; Prado et al., 2015). Importantly, avirulent members belonged to the *Splendidus* group have been shown to participate in quorum sensing resulting in increased virulence of pathogenic strains (De Decker et al., 2013), suggestive that roles of *Vibrio* in bacillary necrosis can be indirect.

6.2.2 How does bacillary necrosis occur- an infectivity model

Even though bacillary necrosis had long been demonstrated to have close links to bacteria (Guillard, 1959), correlation of the microbial community with disease

development in larval cultures has been unsuccessful until this study. For the first time correlations could be made between bacterial count data (Chapter 2) and non-culturable community data (Chapter 3) alongside larval health status. The findings clearly demonstrated that bacillary necrosis involves a systematic shifts in seawater and larval communities as the disease develops and subsides.

Within the culture systems used here, bacillary necrosis is a condition associated with abnormal compositional changes in seawater bacterial communities that can influence the larval bacterial communities and associated health outcomes (Figure 6-1). The abnormal shifts in response to suboptimal rearing conditions may suggest bacillary necrosis can arise from imbalance bacterial communities and does not appear to be associated with any individual bacteria, a situation resembling dysbiosis (Egan and Gardiner, 2016). This would challenge the conventional view on bacillary necrosis that has been based on single pathogen-larval interaction. The importance of imbalanced bacterial communities rather than specific pathogens in bacillary necrosis would require renewed thinking of source, predictability and mitigation of the disease.

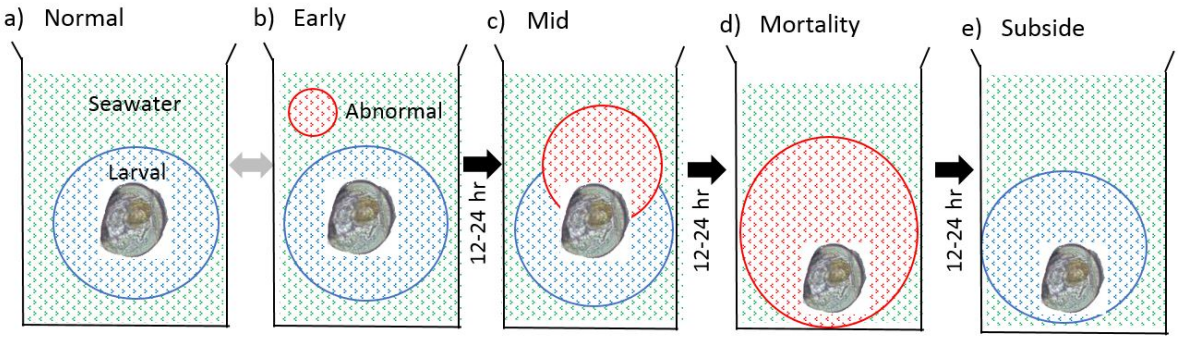


Figure 6-1 Model of development of bacillary necrosis in static larval cultures. Organic enriched condition typical in larval culture can trigger development of abnormal seawater communities unpredictably (b) which can rapidly lead to colonisation of/uptake by larvae (c and d). Green, blue and red dotted areas represent normal seawater, larvae and abnormal bacterial communities. The size of red contour represents the degree of community deviation from those of normal seawater. a-b) Progression can be prevented or reversed by water changes (grey arrow). c) Once larval infection is established the disease is non-reversible by water changes (black arrow). d) Larval mortality is characterised by the convergence of seawater and larval bacterial communities to a specific community. e) Post mortality community is restored to the initial normal state presumably by natural equilibrium.

6.2.3 Sources of infection-contamination versus spontaneity

The majority of commercial hatcheries currently operates on the view that bacillary necrosis is largely caused by accidental introduction or contamination of *Vibrio* or whatever pathogen exists in the production chain (Elston, 1993; Sainz-Hernandez and Maeda-Martinez, 2005; Holbach et al., 2015). This means bacillary necrosis has point sources of infection and intuitively has resulted in stricter hygiene practices throughout the chain of production to ensure low *Vibrio* levels (Elston, 1993). It is unfortunate that only a minority of mortality cases can be correlated to a known source (see Elston et al., 2008b) leaving the majority of cases unresolved. Based on

findings in this thesis, bacillary necrosis is more complex than contamination leading to disease. It instead involves significant bacterial community shifts resulting in abnormal larval-bacterial interactions. For contamination to result in mass mortality, it is suspected the extent of contamination must be significant (such as addition of infected/abnormal bacterial community in algal culture, or bloom of virulent bacteria in intake seawater) that result in rapid displacement of normal communities by sheer quantity of abnormal communities coming into the system. This thesis shows that bacillary necrosis can occur through many possible situations including destabilisation of microbial dynamics in various compartments of the larval rearing systems. Such destabilisation would lead to development of opportunistic communities in the larval cultures, and is influenced by subtle variations or selective pressures that are difficult to control even in smalls scale larval cultures.

The unpredictability of bacillary necrosis raises questions of our current thinking of infection source. The conventional view has been that infection sources of bacillary necrosis are commonly suspected to be biofilms, algal feed, broodstock, larvae itself and seawater (Prado et al., 2005; Eggermont et al., 2014; Dubert et al., 2015). However, very few studies had clearly demonstrated how sources of infection led to bacillary necrosis because seawater bacterial communities are highly transient (Powell et al., 2013) and the fact that contamination does not always lead to bacillary necrosis (Elston et al., 2008b). This thesis argues that seawater naturally contains low levels of diverse genotypes of opportunistic bacteria and the predictability of bacillary necrosis likely lies in how certain bacterial genotypes emerge in response to certain destabilisation. This thesis shows that bacillary necrosis initiates in rearing seawater by development of abnormal communities which rapidly proceed to

larvae where necrosis occurs. Regardless of the infection sources, changes in culture seawater bacterial communities represents an important characteristic in the development of disease. Given that seawater bacterial communities are highly dynamic and diverse (Thompson et al., 2005) depending on the environmental conditions at intake and seawater treatments etc., the emphasis on infection source of pathogens to mitigate such opportunistic pathogenesis fades in comparison with efforts to produce stable microflora in larval cultures.

6.2.4 Why is bacillary necrosis unpredictable?

Bacillary necrosis is often presumed to occur due to pathogens from either uncontrolled contamination and/or major changes in the environment (e.g. Elston et al., 2008a). The highly controlled and replicated larval cultures in Chapter 3 and 4 observed variable occurrence of bacillary necrosis raises the question on what drives this variability of bacillary necrosis.

This thesis indicates that seawater is an important reservoir of diverse bacteria that plays a critical role in the variability of bacillary necrosis onset. To put this into perspective of diversity of seawater bacteria, a study focusing on examining the genotypic diversity of *Vibrio splendidus*, a group that is often associated with bacillary necrosis shows this group consists of at least a thousand distinct genotypes estimated to occur on average at less than one cell per millilitre (Thompson et al., 2005). The sheer diversity of such bacterial group limits the ability of 16S rRNA gene community profiling to confidently resolve most bacteria beyond genus to species level (e.g. Sun et al., 2016), which under represents the genotypic variation that exists in seawater. In chapter 3, larval culture S4 actually had the first rearing

day of normal seawater community as starting inoculum but rapidly deviated to abnormal community in the next 24 h period. This suggests the seawater community characterised using ARISA fingerprinting technique may have missed out the low concentration of genotypes that later emerged and drove the communities to become abnormal. Hence, the link between bacterial diversity and sporadicness is not as easily established due to technical limitation to resolve strain level variation that otherwise could better correlate with sporadicness.

Interestingly, further experiments demonstrated bacillary necrosis remained sporadic despite the use of 0.22 µm filtered seawater in the controlled and replicated larval rearing (reduction in volume to 50 ml and use of algal paste to limit starting bacterial inoculum variation) suggesting that other factors, presumably larval associated bacteria could contribute to variations of bacillary necrosis in larval cultures. This shows the ability of bacillary necrosis to arise from a range of microbial conditions. This could help explain why most studies so far are not able to associate mass mortality episodes with specific bacterial types and have linked with a significantly different bacterial communities in each case (such as Chapman, 2012; Powell et al., 2013).

6.2.5 Monitoring and mitigation of bacillary necrosis

This study furthered our understanding of the usefulness and the limitations of *Vibrio* counts as a routine method to monitor disease in larval cultures. This has relevance because monitoring of *Vibrio* using TCBS plates is still the primary disease monitoring method used in many hatcheries worldwide (e.g. Sainz-Hernandez and Maeda-Martinez, 2005). In this study, initiation of bacillary necrosis was

associated with changes in the culture seawater bacterial community notably as a transient increase in *Vibrio* relative abundance. The increase in *Vibrio* relative abundance means that bacterial plate count monitoring could detect the risk of a bacillary necrosis outbreak. It is worthy of mention that monitoring of major changes in *Vibrio* population is more obvious using the relative *Vibrio* abundance (i.e vibrios/TVC) than *Vibrio* concentration level alone because of the higher level of variability in just the concentration data. Chapter 2 demonstrated larval cultures that suffered bacillary necrosis possessed relatively more abundant *Vibrio* (>20 % of total viable count) in culture seawater 2-3 d prior to the onset of mortality, the time during which seawater communities deviated substantially. Therefore, daily monitoring for relative abundance of *Vibrio* in seawater can detect major changes in seawater community which could mean increased risk of bacillary necrosis outbreak at later date. On the contrary, detection of increased *Vibrio* dominance in larvae is too late as it often coincides with mortality.

The usefulness of *Vibrio* as an early indicator of major bacterial community changes can be attributed to its highly adaptive and fast growth rate (Rehnstam-Holm et al., 2010). However, the rise in *Vibrio* relative abundance was a fraction (<2 % in this thesis) of major shifts in bacterial community which may warrant control for disease not necessarily focused on *Vibrio* alone assuming bacteria that had shifted to achieve a greater dominance are involved in bacillary necrosis. Furthermore, it is shown that *Vibrio* proliferation can also be normal events associated with disease but not all cases of *Vibrio* growth spikes are involved in killing of larvae. It may be helpful that disease management in hatcheries uses *Vibrio* count as a guide to better understand

'behaviour' of bacillary necrosis rather than treating it as a problem.

Currently commercial hatchery operators still lack specific and effective means to mitigate bacillary necrosis partly because of the overall lack of understanding of bacillary necrosis. Water changes are an important part of larval rearing activity mainly because it help reduces organic matter build up in culture therefore controlling the bacterial concentration in larval cultures (Helm et al., 2004). However, there is no direct demonstration of how water changes can help mitigate bacillary necrosis. This study shows 48 h interval water changes currently regarded as a common practice for static culture system (Helm et al., 2004) can be effective if carried out before abnormal microbial changes in the seawater are detected in the larvae (Figure 2-1 and Figure 6-1b). Chapter 3 showed how a larval culture which was developing bacillary necrosis based on the trajectory of bacterial community shifts was successfully mitigated after 2 rounds of 100 % water changes over a 4 d rearing period.

6.3 Characterisation of opportunistic bacteria in mussel larval culture

Chapter 5 contributed to the knowledge of bacillary necrosis in hatchery reared mussel larvae by characterisation of a novel opportunist *Pseudoalteromonas* sp.. This strain belongs to a bacterial group that are ubiquitous and abundant in coastal seawater environment and often associated with marine organisms (see review Bowman, 2007), suggesting potentially more undescribed opportunist pathogens are present in seawater that would constitute normal bacterial flora in the larval culture

environment. It is worthy of mention that opportunistic bacteria may actually present a more complex problem than obligate pathogens because virulence of opportunists are modulated by a variety of factors and therefore more difficult to predict as experienced in Chapter 5. Their presence without causing disease means management of disease against these opportunist are in some ways harder than obligate or true pathogens such as the mostly persistent and highly virulent strains of *V. tubiashii* (Elston et al., 2008b) and *V. splendidus* (Gay et al., 2004a). Detection of opportunist pathogens in the larval rearing environment would not be unexpected and does not necessarily mean disease. The ability of these bacteria to switch from neutral or commensal state to become virulent presents a challenge to current hatchery disease management which is largely based on bacterial load assessment.

Future works should consider understanding conditions under which opportunist *Pseudoalteromonas* T8H10 isolated from this study switches on virulence. This may encompass examinations of growth of the bacterium in the presence of mussel larval host across various parameters (such as nutrient, temperature, salinity, algal feed, stocking density etc.) to simulate larval cultures conditions. Such investigation on more opportunistic bacteria may help build a bigger picture of the opportunistic pathogenesis of bacillary necrosis.

6.4 Further research and development

6.4.1 Causality of bacillary necrosis

A sensible follow-up research is to further understand causality by investigating biological roles of microbes driving the community abnormalities prior to and during onset of mortality. This can be achieved by overlaying microbial community shift data with microbial activity data. However, this would require a more refined and holistic approaches. A metagenomic shotgun sequencing of microbial communities would be the appropriate step. Metagenomic is the study of a collection of over thousands of bacterial genomes from one environmental sample whereas shotgun sequencing is the technique of DNA shearing and sequencing. Instead of using single gene marker (eg. V3 to V5 regions of the 16S rRNA gene as employed in this thesis), the output of bacterial genomes will allow species and potentially genotypic level delineation therefore greatly facilitating high resolution characterisation of bacterial communities (Segata et al., 2012). It is shown in this thesis that amplicon based bacterial community sequencing (i.e metagenetic) is limited in that changes in relative abundance in bacterial genus could not be confidently traced to species or strain levels. Besides, the availability of genome sequence would offer insights into the metabolism, gene functions and lifestyles of bacteria which would help understand the potential interactions with bivalve larvae.

Traditionally, investigations of activity of microbes have always required bacteria to be isolated and cultured. However, the increasingly affordable next generation sequencing (NGS) has also made metatranscriptomic available to environmental studies. This approach sequences gene expression transcripts (i.e RNA) extracted

from environmental samples. This would provide a snap shot of microbial community wide activities and functions without the need of isolating individual strains therefore representing significant time saving and great output of information. Until now, there has never been any metatranscriptomic studies done on bacillary necrosis and findings from this technique is likely going to be novel and would significantly advance our understanding of the nature of opportunistic pathogenesis of this disease.

The combination of metagenomic and metatranscriptomic would help determine key groups of bacteria that are important in the development of disease. These bacteria can be further investigated using various techniques such as quantitative PCR to measure population dynamics and gene expressions, genetic manipulation such as gene knock-out to understand gene functions, multi-bacterial-larval challenge bioassays to examine effect of single and combined strains of bacteria on larval healthy and in-situ hybridisation histopathology to demonstrate cellular interactions.

A major challenge faced in this thesis is inducing bacillary necrosis to produce sufficient samples for analyses. This thesis proposes two ways to improve predictability of disease occurrence: 1) increase number of replicate culture substantially 2) co-inhabitation of healthy larvae in seawater hosting mass mortality. To reduce the logistical complexity of setting up replicate cultures, the use of model larval cultures (as employed in Chapters 2 and 3) can replicate commercial larval cultures but with significantly reduced unrelated environmental variations that can mask observations. Higher number of replicates maybe in the range of one hundred would allow more confident interpretation of association of bacteria, particularly

those that present at lower abundance.

The co-inhabitation of healthy larvae in seawater hosting mass mortality approach is possible because bacillary necrosis observed in this thesis shows bacterial communities shifts from seawater to larvae associated with high mortality events. This approach has been shown to be useful in studying natural infectivity of *Vibrio* bacteria in summer mortality syndromes of spat and adult oysters (Lemire et al., 2015; Petton et al., 2015), allowing researchers to sample more effectively leading up to onset of mortality. Due to the rapid nature of bacillary necrosis, this thesis proposes a minimum of 12 hourly sampling because 24 hourly sampling as used in this study have missed some community changes particularly in the early development of disease and post mortem changes. Shorter sampling interval such as once 6 hourly can be useful if the occurrence of bacillary necrosis can be better predicted (possibly using the aforementioned co-inhabitation approach) and the model larval cultures have enough samples for more frequent samplings.

It is shown in this thesis that mortality is a crude criterion for studying bacillary necrosis. A disease affected larval culture, as determined by abnormal bacterial communities may not show deterioration or death until the disease is fully developed. As a result, care should be exercised in discerning patterns of bacterial community shifts in low mortality cultures as well.

6.4.2 Associations and roles of bacterial protease production

As proposed earlier, the examination of microbial activities in a more thorough fashion using transcriptomic would likely reap new insights. This is because this thesis using simple enzymatic assays has already demonstrated for the first time, association of elevated proteolytic activity in larval culture seawater with high mortality events. The observation of higher proteolytic activity in seawater hosting mass mortalities aligns well with many works proposing secretion of toxic protease as a major virulence factor in many strains of *Vibrio* associated with bacillary necrosis (Le Roux et al., 2007; Binesse et al., 2008; Hasegawa et al., 2008; Hasegawa et al., 2009; Mersni-Achour et al., 2015).

More works are necessary to demonstrate links of elevated proteolytic activities in larval cultures with mass mortalities. This can be investigated for example, by immersing healthy larvae in 0.22 µm filtered seawater that had earlier on showed high proteolytic level seawater and hosted mass mortality. This is to determine if the same seawater can reproduce mortality therefore providing a closer link. Chelating compound such as the ethylenediaminetetraacetic acid (EDTA) can be added to determine if zinc ion dependant metalloprotease, a group of toxic protease most commonly associated with virulent bacteria (Hasegawa et al., 2009; Labreuche et al., 2010) is involved. Secondly, the types of protease in seawater hosting mass mortality need to be characterised. This could be achieved by analysing seawater sample using high performance liquid chromatography (HPLC) and comparison with known protease samples. The diversity of protease may be useful in understanding its sources and substrate specificity (see Wu and Chen, 2011), all of which could help

link specific tissue necrosis in histopathological interpretation with specific groups of bacteria.

6.4.3 How could we create healthy bacterial communities in larval cultures?

Despite being studied for almost half a century, bacillary necrosis unfortunately has yet to be successfully overcome and is hindering bivalve hatchery becoming economically and environmentally sustainable. The understanding of bacillary necrosis has recently started to advance due to improvement of molecular techniques that have helped to unravel the large portion of microbes that were previously unknown. The information on bacteria associated with bacillary necrosis in larval cultures from this thesis provides a framework that will assist the mussel and bivalve hatchery industry in managing the disease to improve production. An in depth understanding of the husbandry practices and its relationship with microbes, and the resultant bacillary necrosis is the basis to effective detection, treatment and prevention of the disease.

The efficacy of current disease detection and mitigation strategy focusing on specific bacteria/pathogens has serious limitations given that bacillary necrosis can be associated with a range of bacteria (see Table 1-1) and is linked with significantly different bacterial communities in each case (such as Chapman, 2012; Powell et al., 2013). This indicates that bacillary necrosis in most cases are not caused by a particular species but can arise from a multitude or microbial assemblages that present opportunistic pathogenesis with reared larvae. Therefore, producing balanced

or beneficial bacterial communities in larval cultures appears logical as a proactive measure to control bacillary necrosis.

Beneficial bacteria can be classified into “neutral bacteria” and bacteria conferring specific benefits known as probiotics. Neutral bacteria constitute the majority of microbes and play important roles in nutrient cycling such as organic matter utilisation and degradation, nitrification, ammonification, nitrate reduction, denitrification and sulphate reduction. These bacteria when allowed to grow and reach stability in a process known as microbial conditioning or maturation, would ensure the maximum carrying capacity of the larval rearing environment is attained therefore leaving less empty niches. This means the available substrate (organic matter) supply per microbe is low resulting in thriving slow growing *K* strategists and suppressed population of fast growing *r* opportunists (Skjermo et al., 1997; Skjermo and Vadstein, 1999). Biological filtration used in recirculation systems have been used with success to favour *K* selection therefore increasing stability, resilience and robustness of the microbial community (Salvesen et al., 1999; Attramadal et al., 2012a).

Another way to create beneficial community is by addition of probiotic cultures to larval cultures. Such bacteria can benefit the host larvae in various ways but the majority of research done on bivalve probiotics so far focus on inhibition of single and rarely a range of pathogens. Several potential probiotics belonging to the genera *Alteromonas*, *Vibrio* and *Roseobacter* were described for Pacific oysters, scallop, and manilla clam (see review Kesarcodi-Watson et al., 2008). *Alteromonas macleodii* and *Neptunomonas* sp. strains were found to reduce the lethality of *Vibrio* pathogens to

mussels in challenge trials (Kesarcodi-Watson et al., 2010). So far, there is no commercial probiotic products that have been shown to be effective for bivalve larvae warranting the needs to also explore other alternatives. This may include the recently proposed concept of Beneficial Microorganism for Corals (BMC, Peixoto et al., 2017). As opposed to the conventional probiotic strategy which inhibits specific pathogens, the BMC proposes the use of a consortium of beneficial bacteria isolated from the host and associated environment as a cocktail of probiotics that can help restore microbial homeostasis hence resilience of coral health. Central to this approach is the use of a range of carefully selected bacteria in the probiotic mix to enhance a range of benefits for the microbial communities such as shelters, nutrient cycling, carbon and nutrient sources, antimicrobial activities, and natural phage infections.

6.4.4 Is recirculation aquaculture system the way forward for bivalve larval cultures?

Bivalve larval cultures in most hatcheries use an open circuit water exchange systems meaning old water is not recirculated but replaced with new water whether in batch or continuous format (Helm et al., 2004). Typically, water sourced from costal intakes is particulate filtered, ozone and/or UV bacterial disinfected and used straight away for rearing without any form of microbial conditioning. This reflects priority of the current water changes to keep waste and bacterial load low in cultures but this may have neglected the implications of frequent microbial turnover.

The reasons to allow a lack of conditioning steps in bivalve hatchery water treatment system is not entirely understood but it is presumed to be technically demanding,

not cost effective due to the short larval cycle compared with finfish larvae, physiochemical qualities of seawater being superior over recirculated water, and possibly there is a perception of inefficiency in controlling bacillary necrosis. Evidence around this needs to be established as seawater maturation achieved in recirculation aquaculture system (RAS) in marine finfish larval rearing showed a notably more stable microbial community that is resistant to opportunistic bacterial invasion and produced overall more healthy and marked improved survival compared with an open circuit system (Skjermo et al., 1997; Salvesen et al., 1999; Skjermo and Vadstein, 1999; Olafsen, 2001). The benefits of producing stable communities in bivalve larval cultures to maintain microbial homeostasis hence promote appropriate larval-bacteria relationship is deemed highly important because bivalve larval bacterial communities can be influenced by abnormal seawater communities as shown in this thesis. Unlike in fish, bivalve larvae possess minimal levels of selection for host associated bacteria meaning their health is vulnerable to the disorders in the ambient seawater bacteria.

The potential of RAS for bivalve larval rearing as an alternative to the open circuit system to create better larval-bacteria relationships is proposed here because it promotes and maintains a strong “neutral” bacterial population, an approach that can manage the issue central to bacillary necrosis i.e. abnormal bacterial communities. There are limited works that investigated suitability of RAS to replace current open circuit rearing system but the findings are inconsistent when it comes to survival, growth rate and competency to metamorphosis. (Magnesen and Jacobsen, 2012; Asmani et al., 2016; Kamermans et al., 2016). Future research is needed to determine its usefulness in overcoming microbial instability associated with seawater and

how it can suppress bacillary necrosis. Data from such studies can be compared for the microbial patterns found in this thesis to establish preliminary difference and potential benefits of such systems. Research in this area also needs to provide understanding around the balance between waste removal and conserving bacteria for a stable microbial communities. Even though physiochemical chemicals such as dissolved oxygen, particulates and DOM in open circuit cultures tanks are superior compared with recirculated water, it is generally accepted that marine larvae survival are more susceptible to bacterial infection provided physiological parameters are not excessively breached.

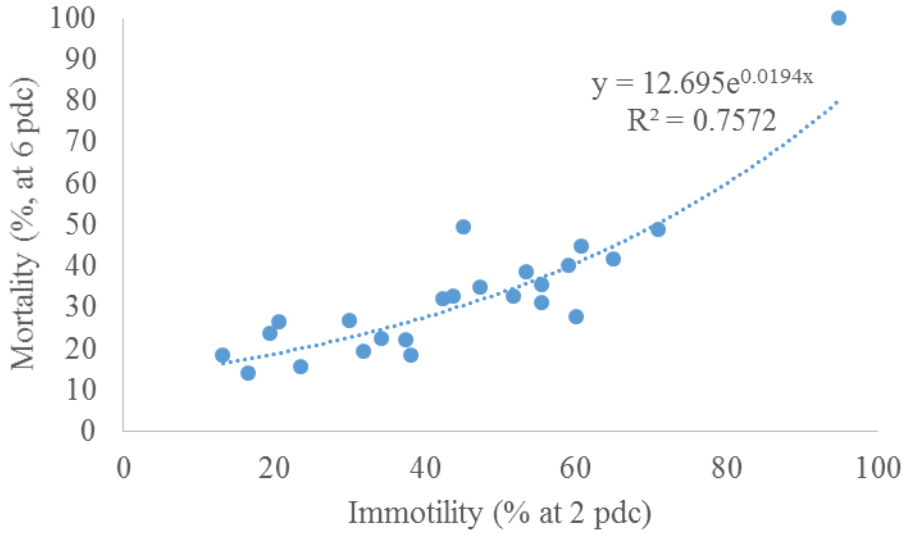
6.5 Concluding remarks

This thesis has added to our understanding of bacillary necrosis by establishing close association of increased relative abundance of *Vibrio* with the development of bacillary necrosis prior to mortality and the demonstration of a novel opportunistic pathogen of *Pseudoalteromonas*, a bacterial genus which has never been reported to affect hatchery larval culture of mussel specifically Australian blue mussel. Finally, this thesis has contributed significantly to the understanding of causation of bacillary necrosis by demonstration of systematic bacterial community changes suggestive of a seawater-to-larvae infection which is novel and have never been reported in bivalve culture of any species.

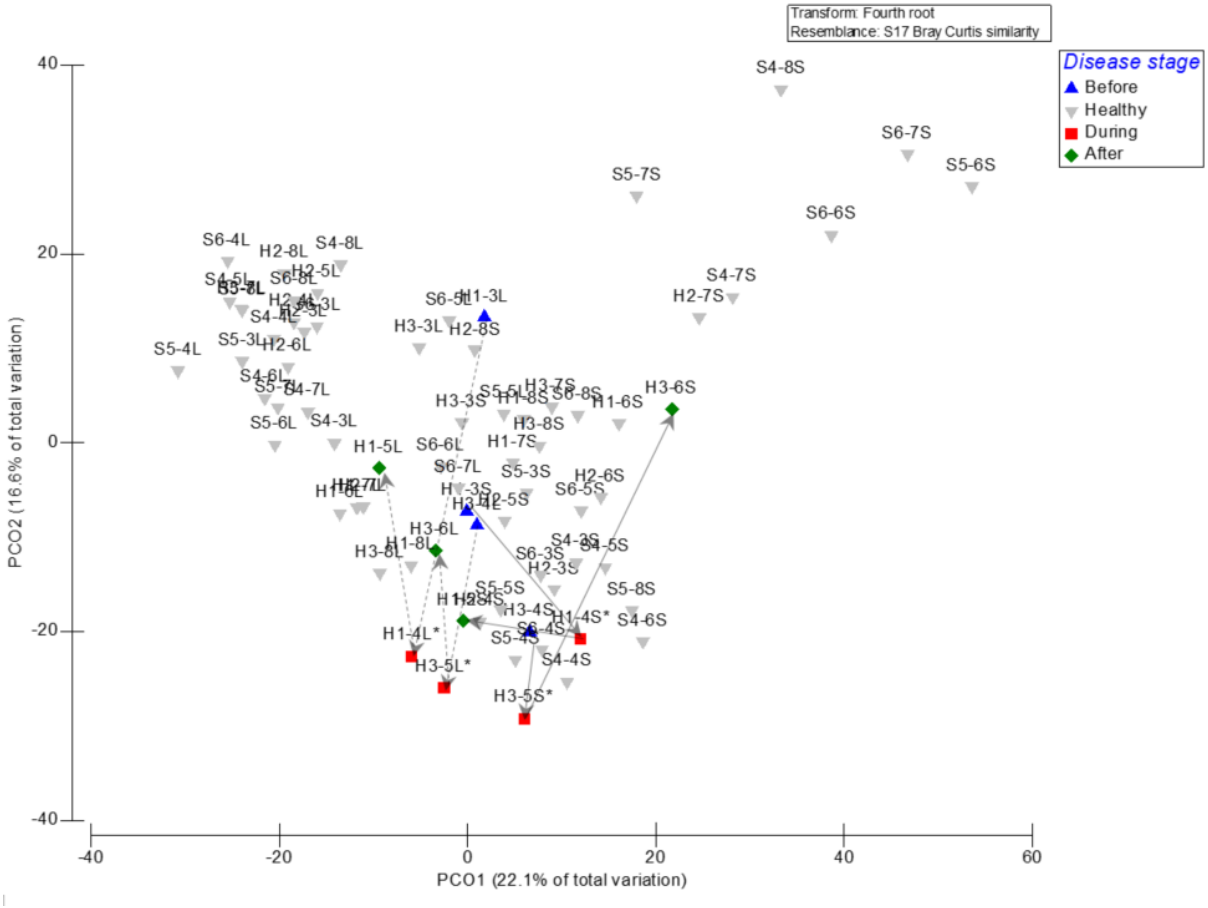
Appendix

Appendix 1. Feeding rate for Australian blue mussel (*Mytilus galloprovincialis*) larval cultures.

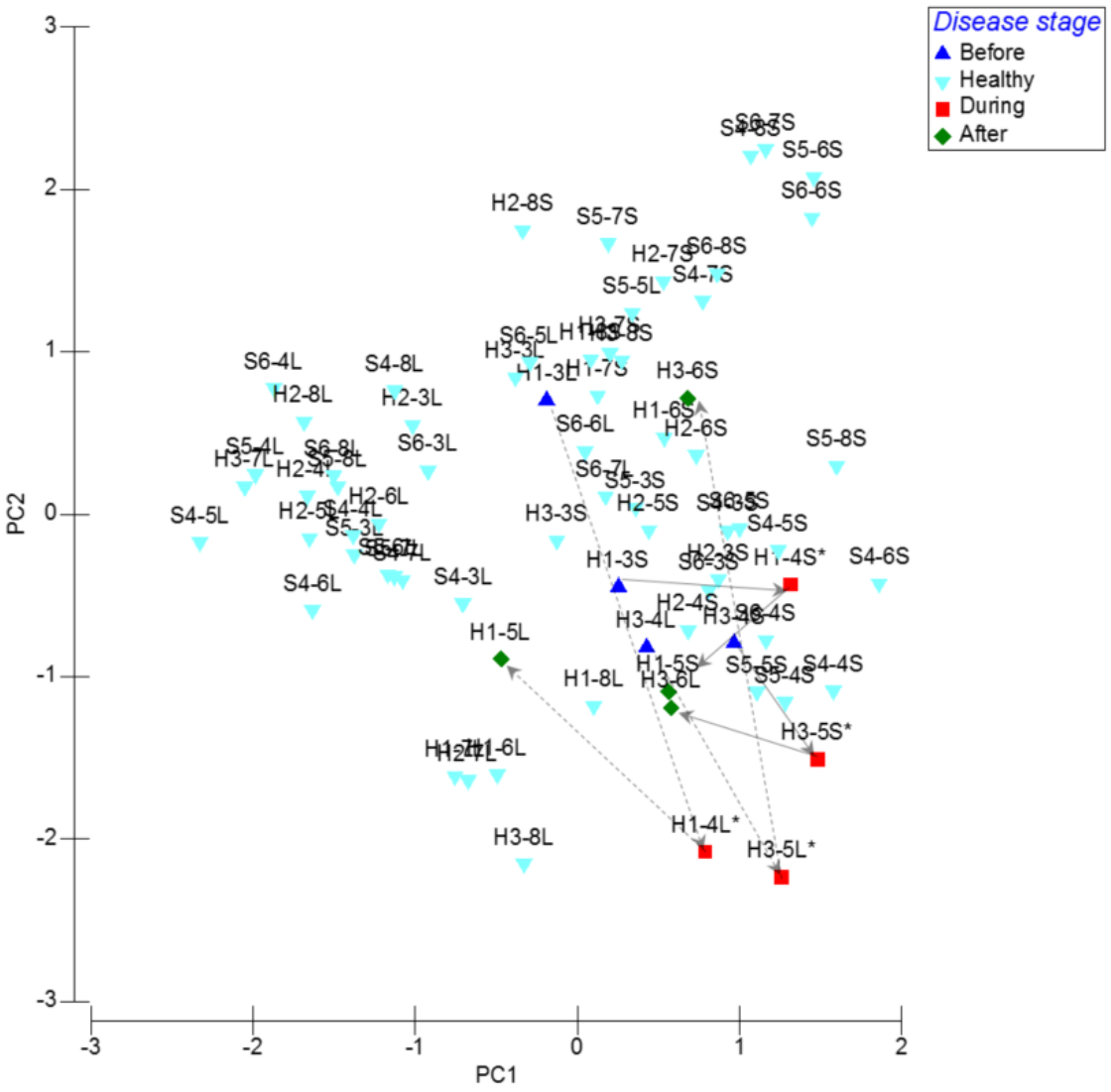
		Feed rate (10^3 cells larva^{-1})		
Age (, dpf)	Size (µm)	*Chaetoceros calcitrans*	**Equivalent** *Isochrysis* spp.	*Equivalent Pavlova lutherii*
1	90	4	2.4	2.4
2	110	7	4.2	4.2
3	120	8.25	4.95	4.95
4	128	11.5	6.9	6.9
5	133	12	7.2	7.2
6	154	13	7.8	7.8
7	170	20	12	12
8	194	31	18.6	18.6
9	214	32	19.2	19.2
10	226	35	21	21
11	256	40	24	24
12	271	50	30	30
13	289	60	36	36
14	300	60	36	36
16	310	60	36	36
18	320	60	36	36
20	330	60	36	36



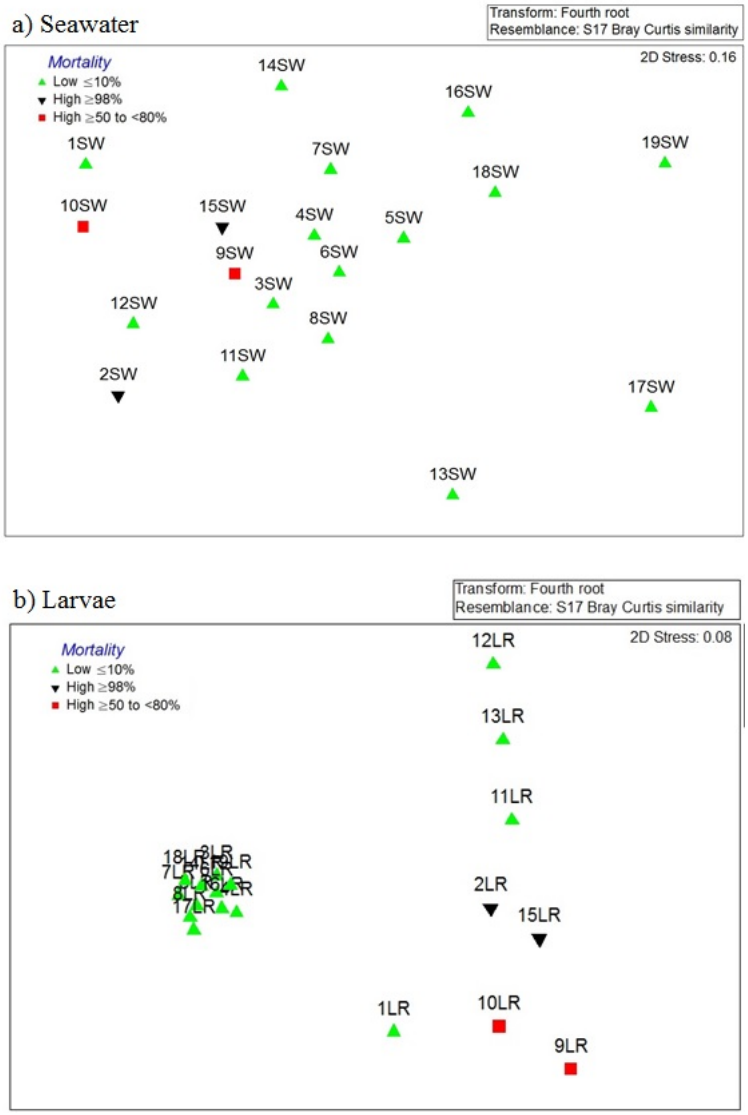
Appendix 2. Scatter plot of percentage of immotility mussel larvae at 22pd with percentage of mortality at 6 dpc. Bioassays were carried out at challenge concentration 10^{7} $CFUml^{-1}$ in triplicate, each used 40 to 50 five dpf larvae in 200 µl of 1 µm filtered seawater. Positive control received oyster pathogen *Vibrio tubiashii* (Isolate ID: 09/2885-1). Negative control received sterile seawater.



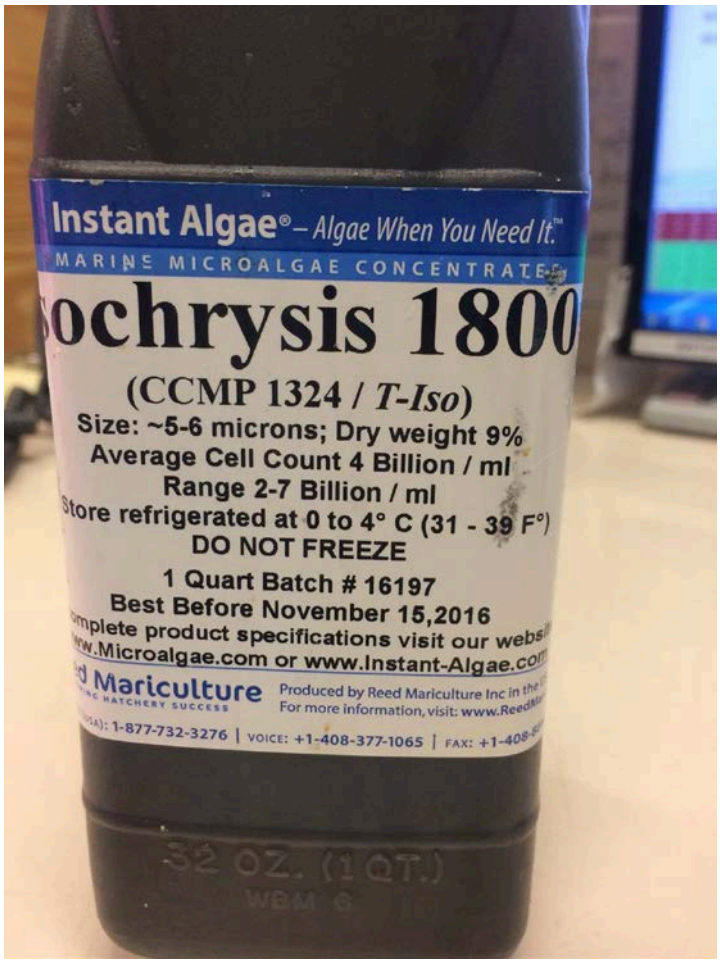
Appendix 3. Principal coordinates (PCO) analysis of all 3 overfed (H1, 2 and 3)and 3 standard fed larval cultures (S4, 5 and 6) reared from3 to 8 dpf (n=72). A total of n=72 ARISA data samples (n=36 seawater (S) and n=36 larvae (L) were included. Principal Coordinate axis 1 and 2 explained 22.1% and 16.6% respectively. Plots associated with mass mortality (n=6) are coloured based on disease stage organised into a day before, during and a day after mass mortality in culture H1 and H3 at 4 and 5 dpf. Culture H2 were normal. Grey plots indicates samples of H1, H2 and H3 that are otherwise associated with healthy larvae.



Appendix 4. Principal component analysis (PCA) analysis of all 3 overfed (H1, 2 and 3) and 3 standard fed larval cultures (S4, 5 and 6) reared from3 to 8 dpf (n=72). A total of n=72 ARISA data samples (n=36 seawater (S) and n=36 larvae (L) were included. Plots associated with mass mortality (n=6) are coloured based on disease stage organised into a day before, during and a day after mass mortality in culture H1 and H3 at 4 and 5 dpf. Culture H2 were normal. Grey plots indicates samples of H1, H2 and H3 that are otherwise associated with healthy larvae.



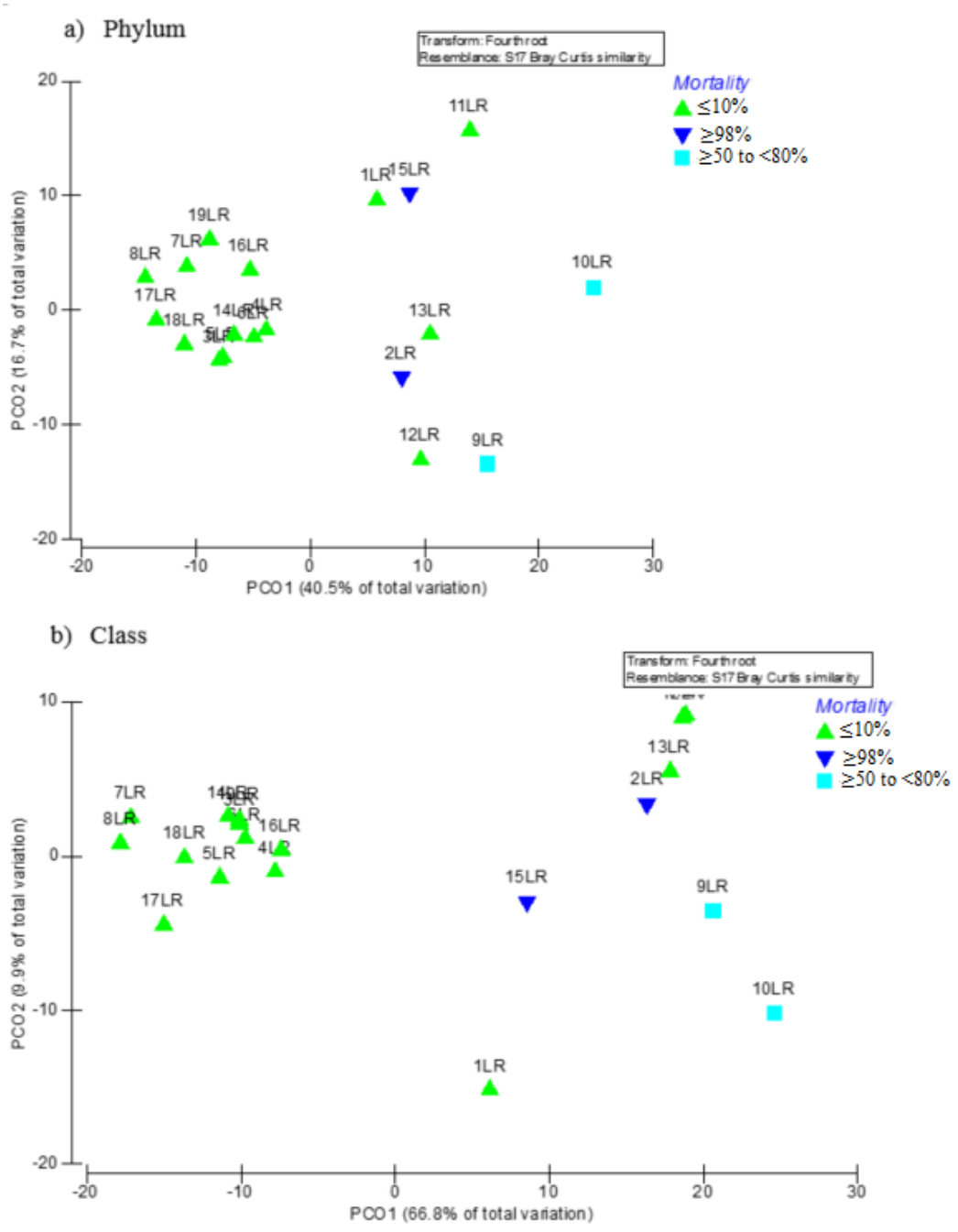
Appendix 5. Multi-dimensional scale (MDS) of a) seawater and b) larval bacterial communities associated with low (≤10%) and high (≥50 to <80%, and ≥98%) mortality.



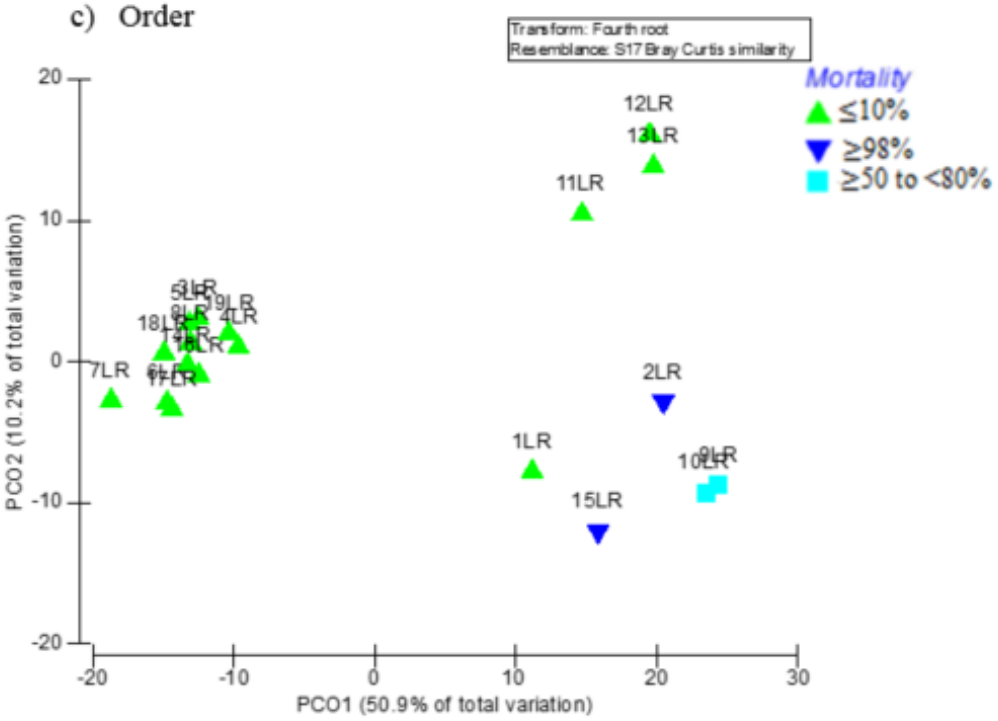
Appendix 6. Commercial *Isochrysis* Tahitian (*T. iso*) paste (Instant Algae, Reed Mariculture, US

Appendix 7. Sequence reads of bacteria were adjusted using median 16S gene copy number available at https://rrndb.umms.med.umich.edu/search/

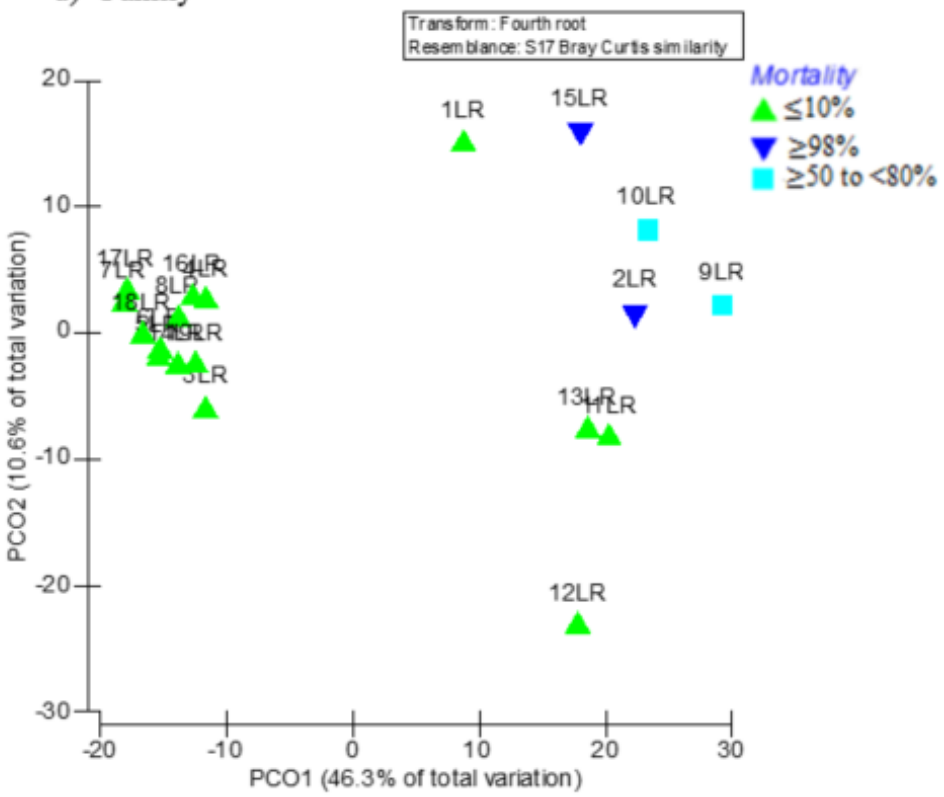
		16S copy	
Genus	Genome sequencing	Range	Median
Alcanivorax	2	2 to 3	2.5
Aliivibrio	2	7 to 12	9.5
Alteromonas	16	5 to 9	5
Arcobacter	5	4 to 5	5
Burkholderia	123	1 to 9	4
Carnobacterium	3	6 to 8	8
Glaciecola	3	4 to 6	5
Leptospira	16	2 to 2	2
Marinomonas	3	7 to 8	8
Methylobacterium	12	4 to 11	5
Moritella	1	11	11
Mycoplasma	68	1 to 2	2
Olleya	1	2	2
Phaeobacter	5	2 to 4	4
Photobacterium	1	15	15
Polaribacter	1	2	2
Propionibacterium	12	2 to 4	3
Pseudoaltermomonas	3	5 to 9	8
Pseudomonas	90	1 to 7	4
Psychroflexus	1	3	3
Sphingomonas	10	1 to 3	2
Shewanella	24	8 to 14	9.5
Sulfitobacter	6	2 to 8	6
Tenacibaculum	2	3 to 10	6.5
Vibrio	79	1 to 15	8
Weissella	4	5 to 6	6
Winogradskyella	1	2	2

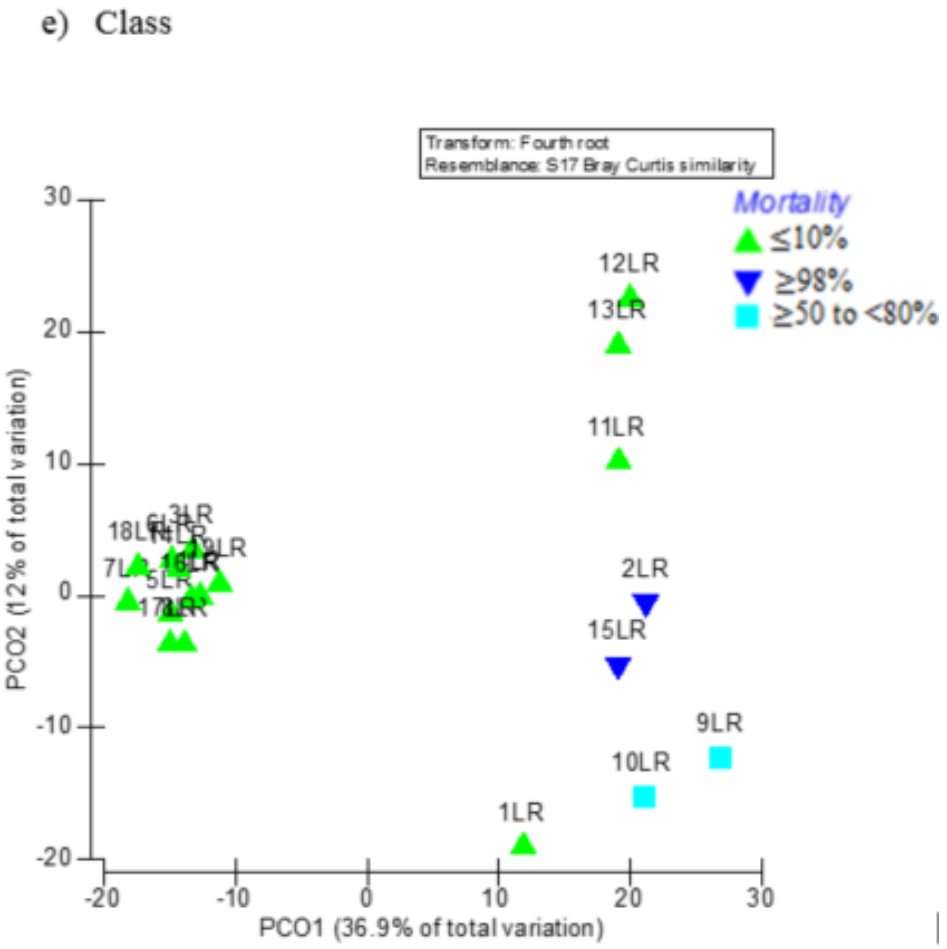

a) Phylum
Transform: Fourth root
Resemblance: S17 Bray Curtis similarity
Mortality
≤10%
≥98%
≥50 to <80%
11LR
1LR
15LR
19LR
7LR
16LR
8LR
10LR
17LR
18LR
13LR
2LR
12LR
9LR
PCO2 (16.7% of total variation)
PCO1 (40.5% of total variation)
b) Class
Transform: Fourth root
Resemblance: S17 Bray Curtis similarity
Mortality
≤10%
≥98%
≥50 to <80%
13LR
2LR
7LR
8LR
18LR
16LR
5LR
17LR
15LR
9LR
10LR
1LR
PCO2 (9.9% of total variation)
PCO1 (66.8% of total variation)

c) Order

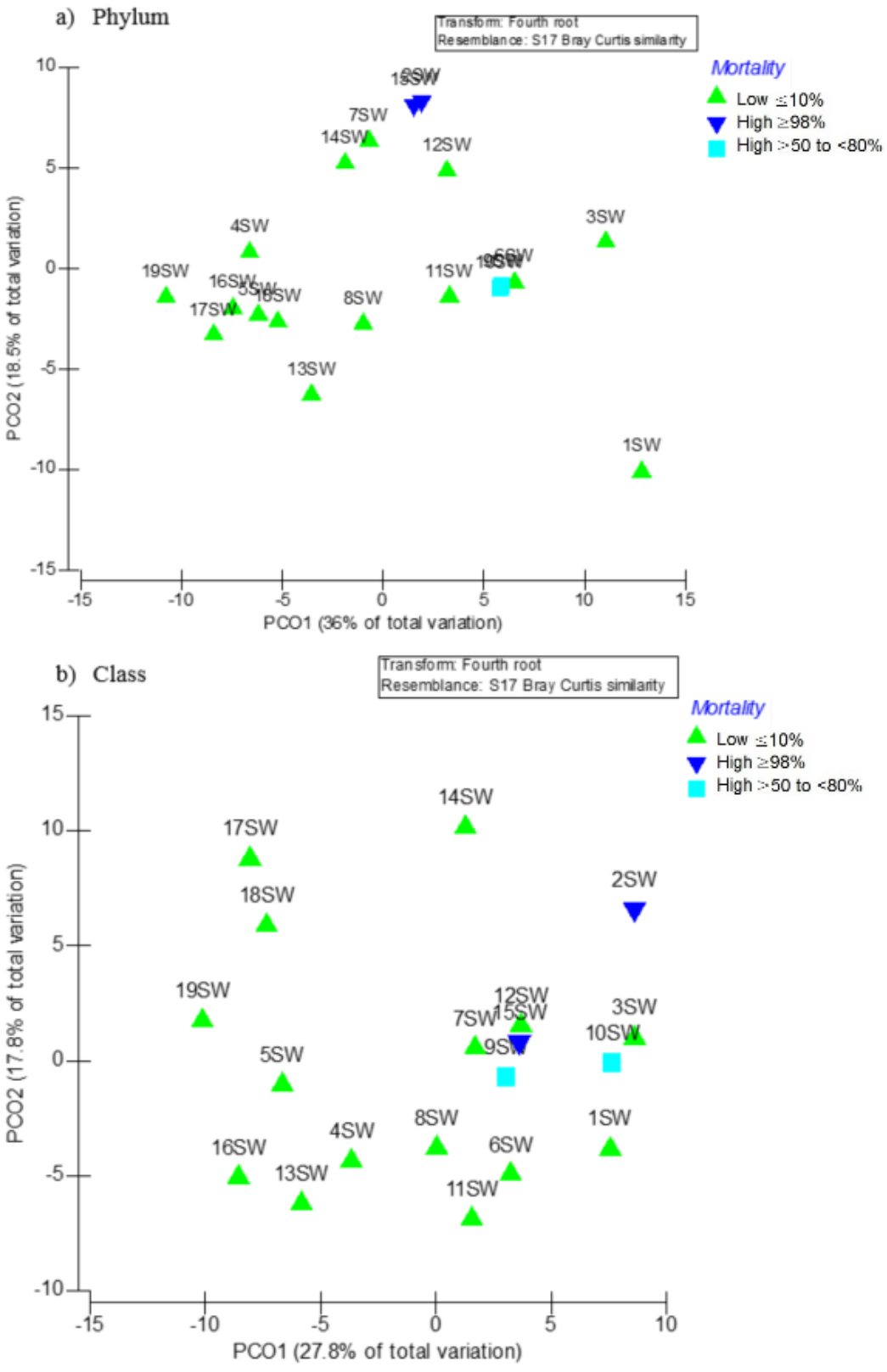


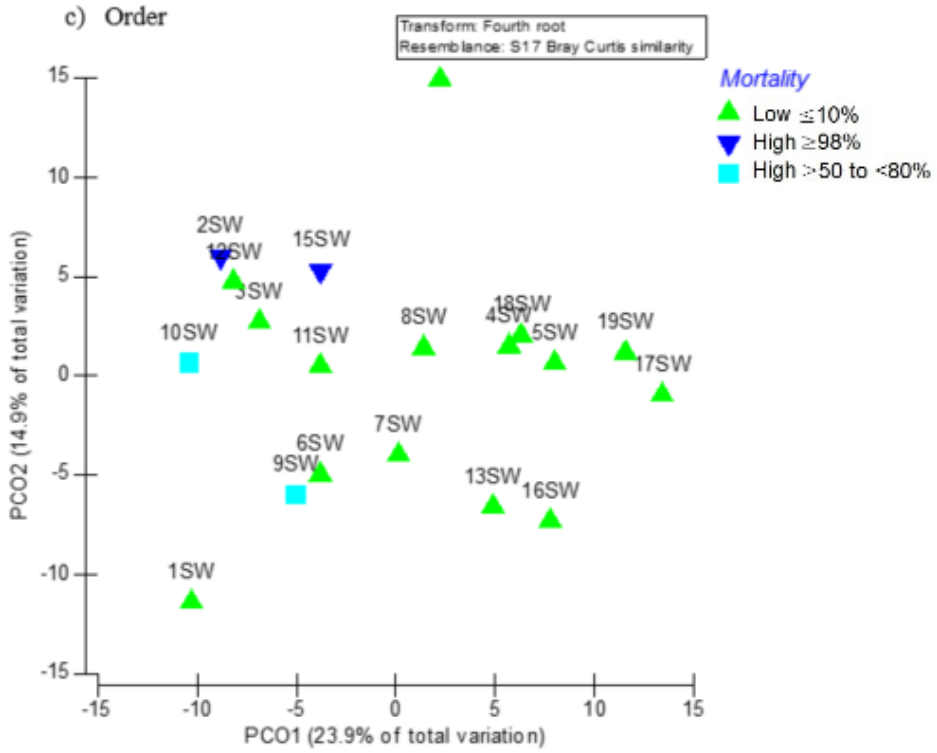
d) Family

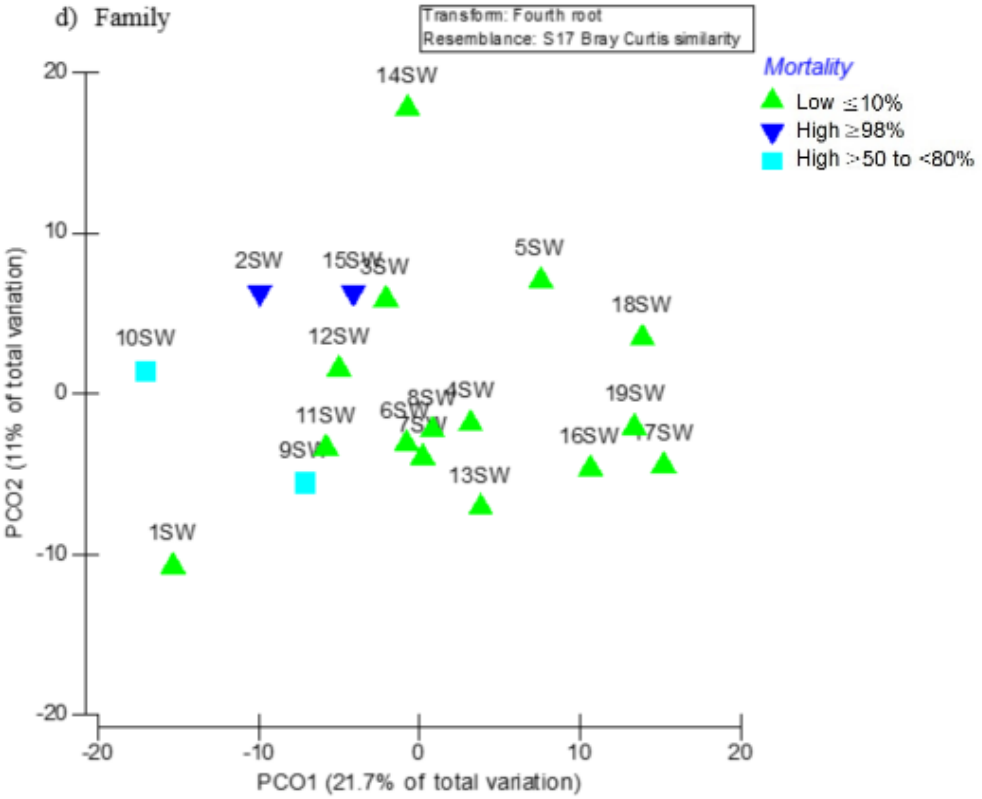


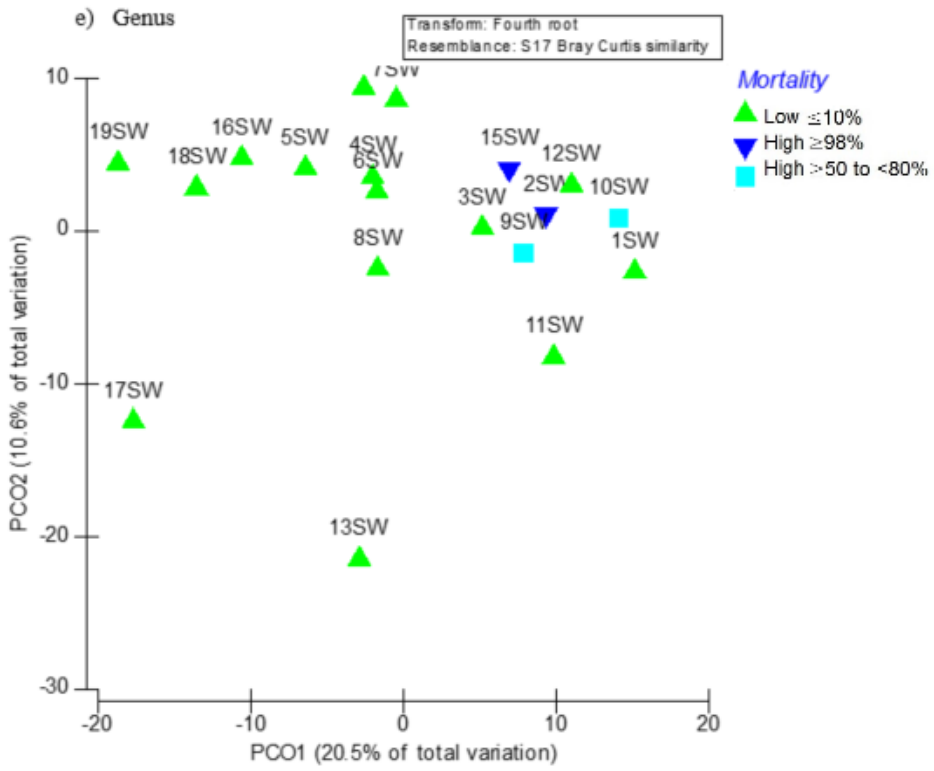


Appendix 8. Principal coordinate (PCO) plots for larval associated bacterial community at a) phylum, b) class, c) order, d) family and e) genus level organised based on mortality level.


a) Phylum
Transform: Fourth root
Resemblance: S17 Bray Curtis similarity
Mortality
Low ≤10%
High ≥98%
High >50 to <80%
PCO2 (18.5% of total variation)
PCO1 (36% of total variation)
b) Class
Transform: Fourth root
Resemblance: S17 Bray Curtis similarity
Mortality
Low ≤10%
High ≥98%
High >50 to <80%
PCO2 (17.8% of total variation)
PCO1 (27.8% of total variation)

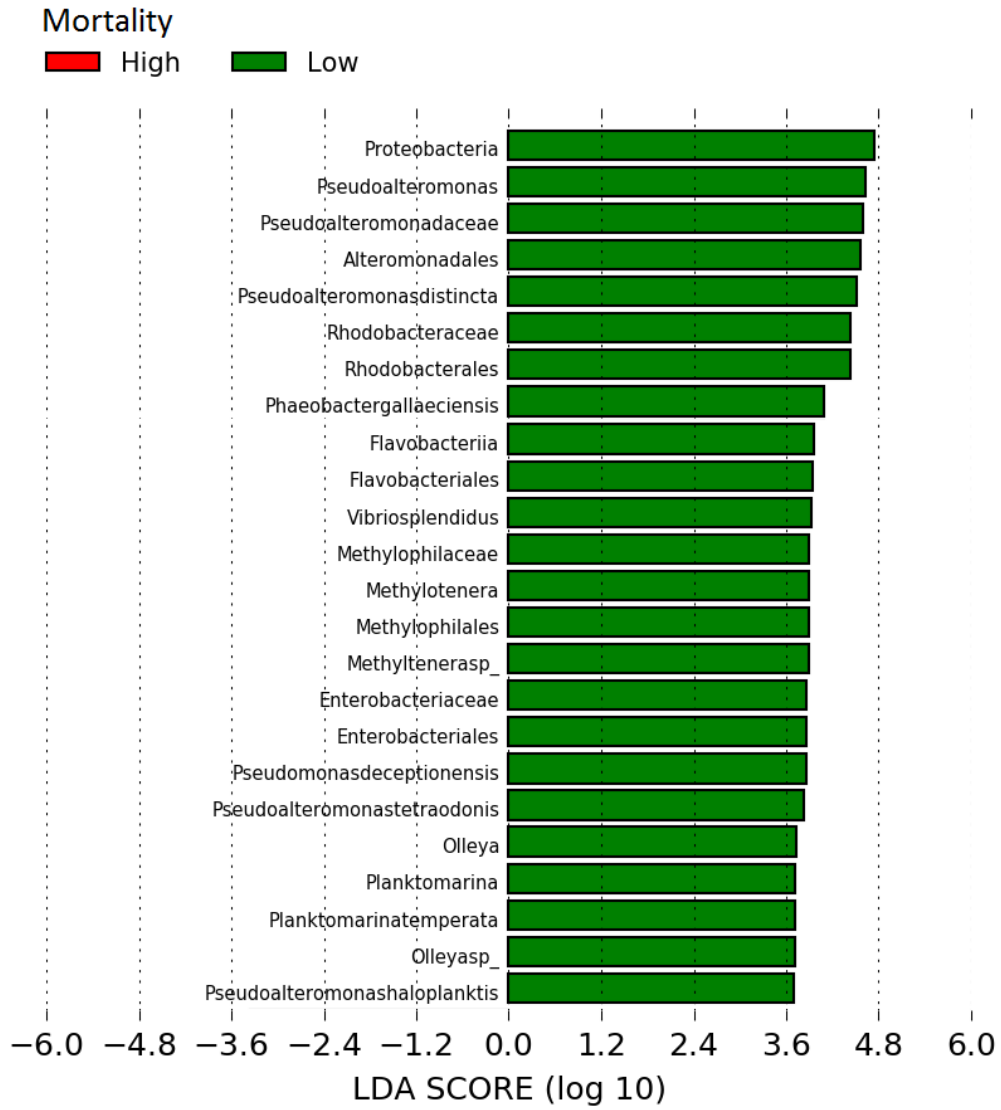

c) Order
Transform: Fourth root
Resemblance: S17 Bray Curtis similarity
Mortality
Low ≤10%
High ≥98%
High >50 to <80%
PCO2 (14.9% of total variation)
PCO1 (23.9% of total variation)
2SW
15SW
3SW
10SW
11SW
8SW
4SW
5SW
19SW
17SW
7SW
6SW
9SW
13SW
16SW
1SW


d) Family
Transform: Fourth root
Resemblance: S17 Bray Curtis similarity
Mortality
Low ≤10%
High ≥98%
High >50 to <80%
PCO2 (11% of total variation)
PCO1 (21.7% of total variation)
14SW
2SW
15SW
3SW
5SW
18SW
10SW
12SW
4SW
19SW
11SW
6SW
8SW
7SW
16SW
17SW
9SW
13SW
1SW

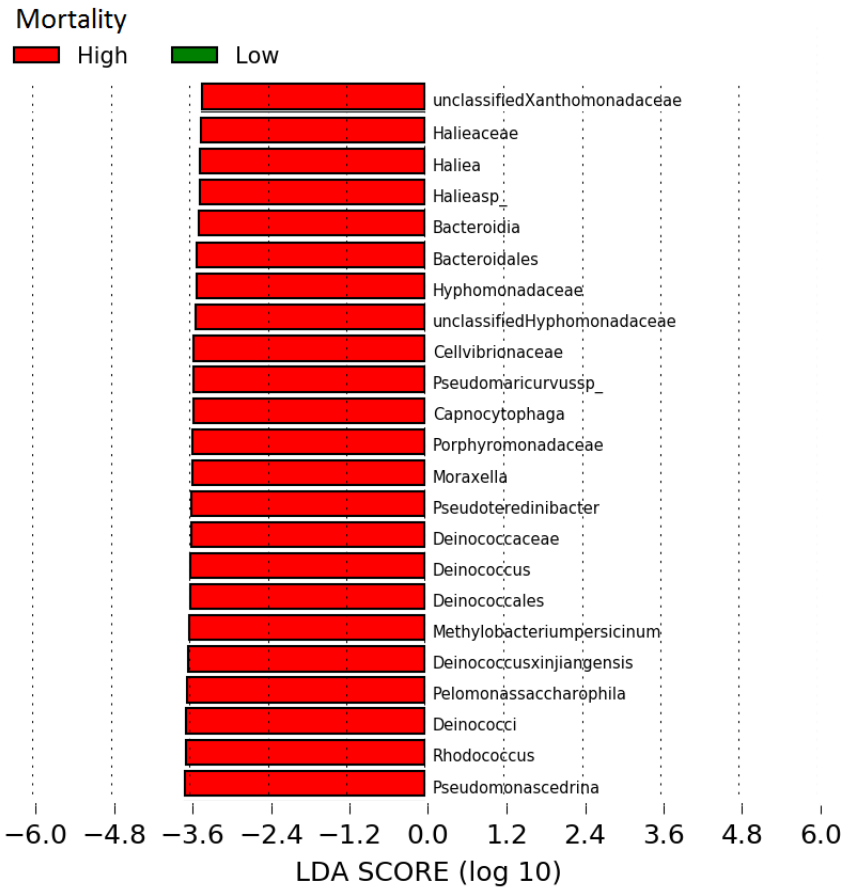


Appendix 9. Principal coordinate (PCO) plots for seawater associated bacterial community at a) phylum, b) class, c) order, d) family and e) genus level organised based on mortality level.

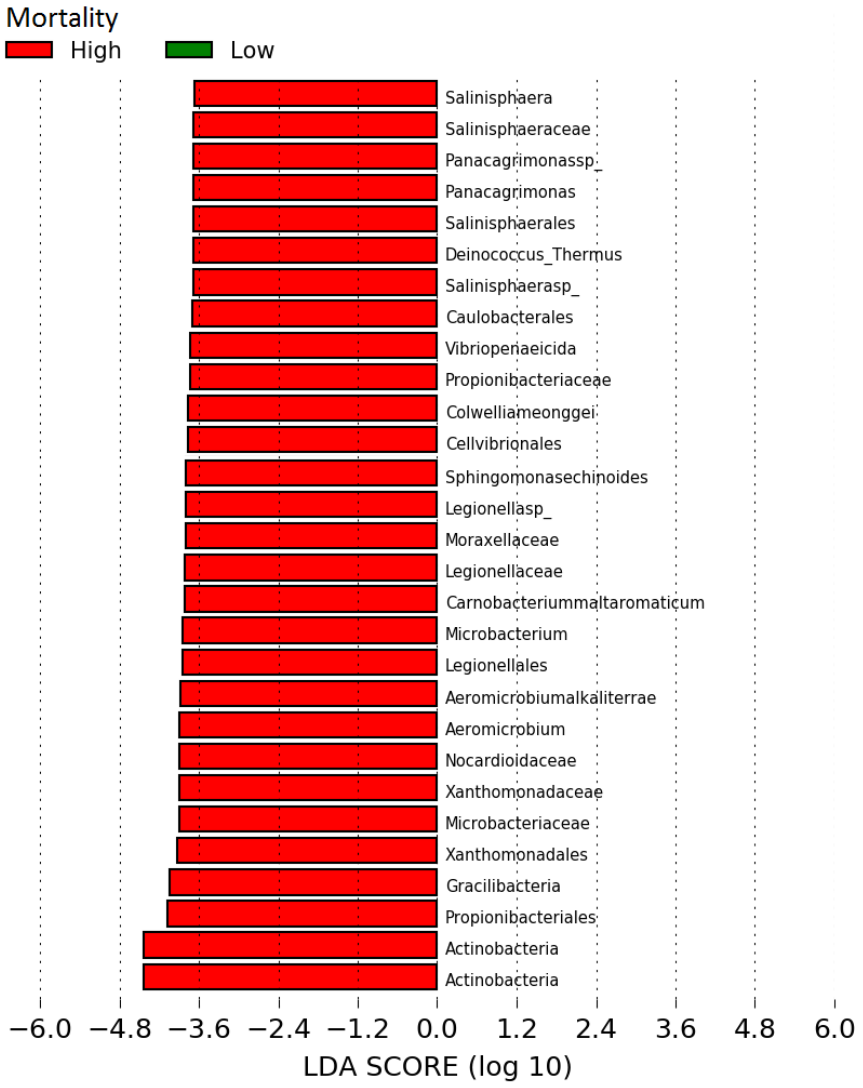
a) Linear discriminant analysis (LDA) score for larval bacterial taxa associated with low mortality (continued)



b) Linear discriminant analysis (LDA) score for larval bacterial taxa associated with high mortality



(continued)



Appendix 10. Linear discriminant analysis (LDA) score for larval bacterial community associated with a) low and b) high mortality.

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